Comparative Echolocation Studies on Bats Using the High Duty Cycle Flutter Detecting Strategy

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List of abbreviations

CF = constant-frequency component
CF_2 = constant-frequency component of the second harmonic
CRF = cochlear resonance frequency
DC = duty cycle
DS = Doppler shift
DSC = Doppler shift compensation
f_{echo} = echo frequency
FM = frequency-modulated component
f_{max} = frequency maximum
f_m = microphone frequency
f_{ref} = reference frequency
f_{rest} = resting frequency
f_s = CF_2 of the emitted signals
iFM = initial frequency-modulated component
hd = halving distance
rms = root mean square
SPL = sound pressure level
tFM = terminal frequency-modulated component
t_{max} = relative skin temperature maximum
v_b = flight speed of the bat

List of terms

Duty cycle

Percentage of time filled by signals calculated from signal duration and the pulse interval:

$$\text{Duty cycle} = \frac{\text{signal duration (ms)}}{\text{pulse interval (ms)}} * 100\ \%$$

Flutter information

Frequency and amplitude modulations in the rhythm of the wing beat in echoes returning from a fluttering insect.

Glint

Strong and short amplitude and spectral peak in the echo, which is produced in the instant where the beating wing of an insect is perpendicular to the impigning sound wave.

$Q_{10\ dB}$ value

The $Q_{10\ dB}$ value indicates the tuning properties of an auditory neuron. The higher the value, the more narrow the tuning to a specific frequency. It is measured as the relation between best frequency of the tuning curve and its width of the neuron 10 dB above.

Reference frequency

Measured in DSC bats as averaged CF_2 in echoes from ahead.

Resting frequency

Measured in stationary bats as average CF_2 of the emitted signals.

Zusammenfassung

Fledermäuse der Gilde „narrow space flutter detecting foragers“ besitzen ein hochspezialisiertes Echoortungssystem, um flatternde Beute im „narrow space“ jagen zu können. Dafür nutzen sie eine lange, konstant-frequente Komponente ihrer Ortungssignale, mit der Hauptintensität in der 2. Harmonischen (CF_2). Die Flügelschlagbewegungen der Insekten erzeugen rhythmische Frequenz- und Amplitudenmodulationen in CF_2 der Echos, was eine Unterscheidung von nicht modulierten Hintergrundechos ermöglicht. Sender und Empfänger sind speziell an das Dekodieren von Flatterinformation aus dem Echo angepasst. Über audio-vokale Kontrolle senkt der Sender die ausgesandte Frequenz während eines Fluges so ab, dass die Echofrequenz bei einer Referenzfrequenz (f_{ref}) konstant gehalten wird und dadurch Doppler-Effekte in Echos aus der Vorausrichtung kompensiert werden. Dementsprechend wird die Trägerfrequenz der Insektenechos in der Nähe von f_{ref} gehalten. Auf der Empfängerseite garantiert die auditorische Fovea, eine Überrepräsentation von Frequenzen auf der Basilarmembran im Bereich von f_{ref}, dass in den nachgeschalteten fovealen Gebieten der Hörbahn, die Flatterinformation dekodiert und im audio-vokalen System die Echofrequenz bei f_{ref} eingestellt werden kann. Das Audiogramm besitzt ein scharf abgestimmtes Minimum im Bereich der f_{ref}. Die genaue Abstimmung des Senders und des Empfängers auf f_{ref} ist nur in wenigen, repräsentativen Arten untersucht, wird jedoch für alle Vertreter dieser Gilde (~200 Arten) angenommen. Da diese Abstimmung zwingend notwendig ist, um Flatterbewegungen detektieren und analysieren zu können, wird in der vorliegenden Arbeit bei zwei weiteren Arten untersucht, wie Sender und Empfänger aufeinander angepasst sind.

Die Arbeit besteht aus zwei Kapiteln. Ein Kapitel ist bereits publiziert, das andere Kapitel ist ein Manuskript, das demnächst eingereicht werden soll.

Im ersten schon publizierten Kapitel [Schoeppler, D., Denzinger, A. and Schnitzler, H.-U., 2022: The resting frequency of echolocation signals changes with body temperature in the hipposiderid bat *Hipposideros armiger*. *J. Exp. Biol.* 225(3), jeb243569] wurde für die

Rundblattnase *Hipposideros armiger* gezeigt, wie sich die zirkadiane Variation der Körpertemperatur auf CF_2 auswirkt. Dazu wurde die Hauttemperatur mittels eines Miniatur-Temperaturloggers auf dem Rücken der Tiere gemessen. Parallel dazu nahmen wir die Ortungsrufe auf. Für die Analyse konzentrierten wir uns auf induzierte Aktivierungsphasen aus dem Torpor, wo neben großen Temperaturänderungen kontinuierlich Ortungsrufe ausgesandt wurden. Wenn *H. armiger* die Körpertemperatur nach einer Aktivierung aus dem Torpor erhöhte, ging dies immer auch mit einer Erhöhung der Ruhefrequenz, um bis zu 1.44 kHz einher. Daraus lässt sich schließen, dass die Änderung der Körpertemperatur einen deutlichen Effekt auf den Aktivierungszustand des fovealen Resonanzbereichs der Cochlea hatte, was zu der beobachteten Änderung der Aussendefrequenz führte. Die Folgerungen aus diesen Ergebnissen für die Organisation des audio-vokalen Kontrollsystems werden für alle Vertreter dieser Gilde diskutiert.

Das zweite Kapitel wurde als Manuskript verfasst. Es umfasst meinen eigenen Teil, die Messung der Doppler-Effekt Kompensation (DSC) von *R. paradoxolophus*, ergänzt durch die Ergebnisse der Diplomarbeit von Katrin Kost (geb. Schmuck) mit dem Titel „Die Pinnarreflexschwelle bei *Rhinolophus paradoxolophus*". Das Manuskript mit dem Titel "Doppler shift compensation and audiogram of the low frequency horseshoe bat *Rhinolophus paradoxolophus* are comparable to that of other rhinolophids" von den Autoren Schoeppler, D., Kost, K., Schnitzler, H.-U. und Denzinger, A. soll demnächst zur Publikation eingereicht werden. In diesem Kapitel wird gezeigt, dass sowohl der Sender, als auch der Empfänger der morphologisch auffälligen Hufeisennase *Rhinolophus paradoxolophus*, die auch bei der Echoortung auffällt, weil sie eine Oktave tiefer ruft als bei ihrer Körpergröße zu erwarten wäre, ähnlich organisiert sind wie es für alle Vertreter der „flutter detecting foragers" angenommen wird. Die Qualität der DSC, gemessen an der Fähigkeit f_{ref} einzuhalten, ist vergleichbar mit der anderer „flutter detecting foragers". Die foveale Organisation des Hörsystems ist erkennbar durch das Minimum im Audiogram, welches auf die f_{ref} abgestimmt ist. Die f_{ref} ergibt sich aus der Kopplung der f_{ref} und der f_{rest}. Diese Übereinstimmung zeigt, dass das tieffrequente Echoortungssystem von *R. paradoxolophus* wie erwartet an die Auswertung von Flatterinformation angepasst ist.

Beide Arbeiten zeigen, dass bei den untersuchten Arten Sender und Empfänger präzise aufeinander abgestimmt sind, was als Voraussetzung für die Detektion und Analyse von Flatterinformation in den Echos von flatternden Insekten postuliert wird. Dies trifft sowohl auf das tieffrequente Echoortungssystem der Außenseiter-Hufeisennase *Rhinolophus paradoxolophus* zu, als auch auf das Echoortungssystem von *Hipposideros armiger*, welches durch Nachführen der Aussendefrequenz temperaturbedingte Änderungen im fovealen Resonanzbereich der Cochlea ausgleicht.

General introduction

Bat guilds

The combination of powered flight and laryngeal echolocation facilitated a high diversity of 'microbats', accounting for about 1200 species of all chiropterans (~1400 species: Wilson and Mittermeier, 2019). During evolution, echolocation systems and flight morphology have been adapted to the tasks bats have to perform for when acquiring food and orienting in space (Schnitzler et al., 2003; Denzinger and Schnitzler, 2013). Thereby, similar constraints depending on the type of foraging habitat (open, edge, and narrow space), foraging mode (aerial-hawking, trawling, active gleaning, passive gleaning and flutter detection), and to a lesser degree also on the diet led to similar adaptations in flight morphology and echolocation systems (Denzinger and Schnitzler, 2013). According to similar habitat type and foraging mode, microbats can be classified into functional groups or guilds (Schnitzler and Kalko, 2001; Schnitzler et al., 2003). So far, seven guilds are described (Denzinger and Schnitzler, 2013). About one sixth of all microbats (~200 species: Wilson and Mittermeier, 2019) belong to the guild of "narrow space flutter detecting foragers", which have specialized during evolution to discriminate the frequency and amplitude modulated echoes of fluttering insects from unmodulated background echoes, using the flutter detecting echolocation strategy (Denzinger et al., 2018).

High duty cycle flutter detecting foragers

Members of this guild are the closely related sister taxa of Rhinolophidae and Hipposideridae. Together with 4 other families of microbats and the megabats (non-laryngeal echolocating flying foxes and fruit bats), they belong to the clade of Yinpterochiroptera, which is restricted to the Old World (Teeling et al., 2005). All other laryngeal echolocating bats belong to the sister clade Yangochiroptera. One member of the guild of flutter detecting foragers, the New World mormoopid *Pteronotus parnellii*, is nested within the Yangochiroptera. The high phylogenetic distance to Rhinolophidae and Hipposideridae implies a convergent evolution of the flutter detecting strategy (Jones and Teeling, 2006). In contrast to *P. parnellii*, which emits echolocation signals through the mouth, hipposiderids (Fig. 1A) and rhinolophids (Fig. 1B) emit their signals through the nostrils, which are surrounded by complex noseleaf structures. These structures obviously gave the two families their names and are useful features for species identification.

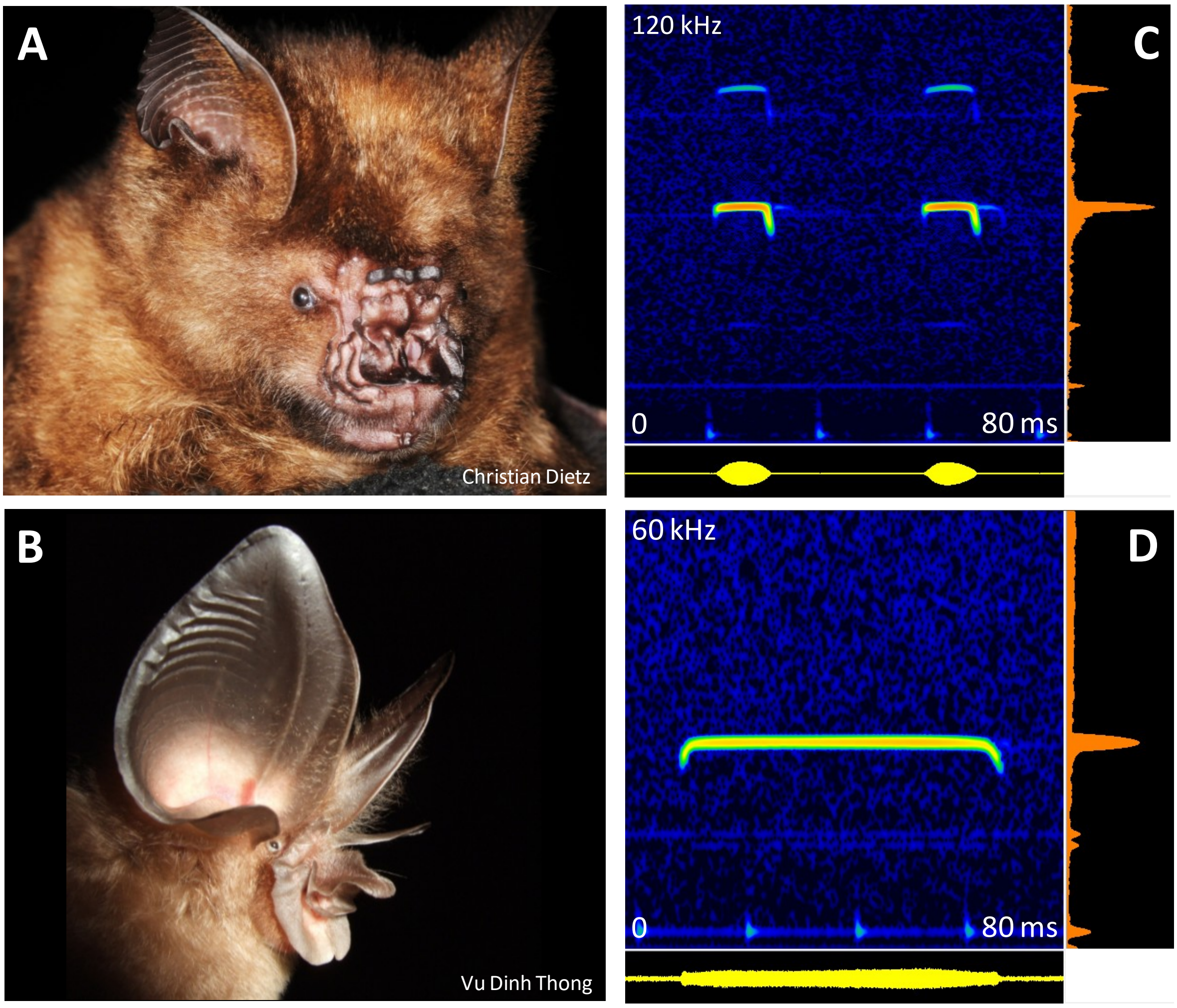

Fig. 1: Portraits and echolocation signals of the two species from this study. (A) The hipposiderid *Hipposideros armiger* (photographed by Christian Dietz) and (B) the rhinolophid *Rhinolophus paradoxolophus* (photographed by Vu Dinh Thong). The echolocation signals (C, D) are recorded in flight and displayed at an FFT of 1024, with the averaged power spectra (right).

The multi-harmonic echolocation signals of flutter detecting foragers are composed of a long constant-frequency component (CF), which is always followed by a short downward frequency-modulated component (tFM) and sometimes preceded by a short upward modulated initial component (iFM) (see Fig. 1D). All bats using the flutter detecting strategy emphasize the second harmonic (CF$_2$) (Fig. 1C, D) and retain the CF in approach phases (Fig. 2) (Schnitzler and Denzinger, 2011). The species-specific CF$_2$ is in an allometric relationship to body size, generally the smaller the bat, the higher the frequency (Heller and Helverson, 1989). The CF$_2$ of hipposiderids is higher than in rhinolophid species of the same size. The duration of echolocation signals ranges from 5-20 ms in hipposiderids (Fig. 1C) to 50-80 ms in rhinolophids (Fig. 1D) (Schnitzler and Denzinger, 2011). Long echolocation signals result in a high duty cycle (DC), which corresponds to the percentage of time filled with signal. Due to this adaptation, bats of this guild are also termed 'high duty cycle bats', which separates them from all other echolocating bats emitting signals at a lower DC of less than 25%, the 'low duty cycle bats' (Fenton et al., 2012).

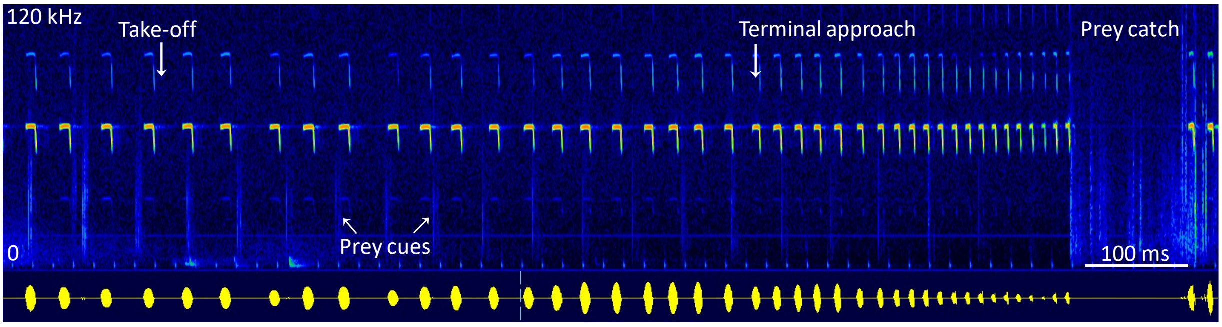

Fig. 2: Echolocation sequence of *Hipposideros armiger* catching a tethered locust in the lab. The tethered prey (*Schistocerca gregaria*) was fluttering at a wing beat rate of 20 Hz. In this example, the wing beats are indicated in the sonogram by rhythmical spectral clicks (prey cues), which are produced when the wings are beating against each other or the string. *Hipposideros armiger* emitted after take-off at a DC of ~30% (search phase or initial approach) and ~40% during the terminal approach.

Flutter information in the echoes returning from fluttering insects consists of rhythmical frequency and amplitude modulations, which are caused by the wing movements of the prey. At each wing beat cycle, a short amplitude and spectral peak, the glint, is produced when the wings are perpendicular to the impinging echolocation signal (reviewed in Schnitzler and Denzinger, 2011).

The transmitter and the receiver of the echolocation system of flutter detection foragers are specialized for the evaluation of flutter information (for reviews, see Schnitzler and Ostwald, 1983; Schnitzler and Denzinger, 2011).

Transmitter and receiver adaptations for the evaluation of flutter information

An adaptation of the transmitter is the Doppler shift compensation (DSC). Flutter detecting foragers keep the CF_2 of echoes (f_{echo}) from targets ahead constant at the so called reference frequency (f_{ref} = average f_{echo}), thereby compensating the Doppler shifts

(DS), which are caused by their own forward movement (Schnitzler, 1968; Schuller, et al., 1974). This f_{ref} is always separated by an offset of up to 200 Hz from the slightly lower resting frequency (f_{rest}), which corresponds to the average CF_2 of signals emitted by resting bats. The ability to keep the two coupled frequencies f_{ref} and f_{rest} constant is with 0.1-0.2% deviation very high (Schnitzler and Denzinger, 2011; Schoeppler et al., 2018). By DSC, flutter detecting foragers adjust the carrier frequency of insect echoes in a frequency range near f_{ref}, where the receiver is adapted for the evaluation of flutter information. This carrier frequency deviates from f_{ref} according to DS caused by the relative speed vector of the flying insects towards or away from the bat (Trappe and Schnitzler, 1982). Another transmitter adaptation is the ability of high DC bats to react to flutter information with an increase in DC (reviewed in Schnitzler and Denzinger, 2011).

An adaptation of the receiver for the evaluation of flutter information is the auditory fovea, a highly expanded frequency representation on the basilar membrane of the cochlea near f_{ref} (for reviews, see Neuweiler et el. 1980; Vater, 1988; Kössl and Vater, 1995). Afferent projections lead to foveal areas in higher centres of the ascending auditory pathway with many sharply tuned neurons, which are specialized for the evaluation of flutter information (for reviews, see Neuweiler et al., 1980; Schnitzler and Denzinger, 2011). The foveal neurons are highly tuned. Their high $Q_{10\,dB}$ values of up to 400 in *R. ferrumequinum* and *P. parnellii* (summarized in Suga and Jen 1977) and up to 140 in *Hipposideros diadema* (Jen and Suthers, 1982) are indicating high frequency selectivity. They project into the flutter processing area of the auditory cortex, where flutter information is extracted (Ostwald 1984; Suga 1984).

The foveal organisation of the hearing system of flutter detecting foragers is also reflected in the behaviourally and electro-physiologically measured audiograms of these bats (e.g., Neuweiler, 1970; Pollak et al., 1972; Suga et al., 1975; Long and Schnitzler, 1975; Schuller, 1980). They are characterized by a distinct minimum near f_{ref} with a steep threshold increase towards lower frequencies up to a threshold maximum, so that the lower emission frequency of DSC bats is attenuated. In addition, there is a threshold increase towards higher frequencies.

Audio-vocal control

The flutter detecting strategy requires that DSC adjusts the CF_2 of echoes with flutter information exactly there, where the auditory fovea produces the highest overrepresentation of sharply tuned flutter evaluating neurons in the foveal areas of the brain. This is the case when f_{ref} is adjusted exactly at the minimum of the audiogram like demonstrated in *R. ferrumequnium*, *R. ferrumequinum nippon*, *R. rouxii*, and *P. parnellii* (Long and Schnitzler, 1975; Schnitzler et al., 1976; Tanuguchi, 1985; Henson et al., 1980, 1985). This match is guaranteed by an audio-vocal control system for DSC, where the activation state of the fovea determines the frequency of the emitted signals (Schoeppler et al., 2018). If the system detects a deviation between the measured state and the desired value (set point), it is initiating a readjustment - a decrease or an increase - of the emission frequency, like in a push and pull mechanism (Metzner et al., 2002; Smotherman et al., 2003).

Open questions

Mainly from studies with the rhinolophids *R. ferrumequinum*, *R. rouxii*, and the mormoopid *P. parnellii*, it is assumed that transmitter and receiver of all flutter detecting foragers are matched by DSC, which adjust echoes from targets ahead at f_{ref}, and an auditory fovea, which is tuned to f_{ref}. This match guarantees that flutter information in echoes from fluttering insects can be decoded by the specialized auditory system. The assumption also predicts that the match should be evident independent whether the CF_2 of high duty cycle bats varies between species, between individuals within one species, but also within individuals.

Aims of chapter 1

Distinct variations of the coupled frequency pair f_{ref} and f_{rest} within individuals have been reported for the mormoopid *P. parnellii* and have been explained by variation of the body temperature (Huffman and Henson, 1991, 1993). However, CF_2 variations in some species of hipposiderids and rhinolophids were related to behavioural contexts (Hiryu et al., 2006; Furusawa et al., 2012). Under the assumption that not only in *P. parnellii*, but also in all flutter detecting foragers, a change in body temperature has a strong effect on the activation state of the fovea, we measured in the hipposiderid bat *Hipposideros armiger*, how the circadian variation of body temperature affected f_{rest}.

Further, we discussed the implications of the temperature-induced variations in f_{rest} for the organization and function of the audio-vocal control systems of all flutter detecting foragers.

The results of this study are presented in the already published paper "The resting frequency of echolocation signals changes with body temperature in the hipposiderid bat *Hipposideros armiger*" (Schoeppler, D., Denzinger, A. and Schnitzler, H.-U., 2022). This publication is the first chapter of my thesis.

Aims of chapter 2

Furthermore, we studied the matching of transmitter and receiver adaptations in *Rhinolophus paradoxolopus*, a rhinolophid outlier characterized by a low frequency echolocation system, with a CF_2 which is about one octave below the frequency of other rhinolophid species of the same size. We hypothesized that the f_{ref} adjusted by DSC should coincide with a minimum of the behavioural audiogram at f_{ref} to guarantee the perception and evaluation of flutter information.

To test this hypothesis, we described the DSC system of *R. paradoxolophus* and measured behavioural audiograms. The results of these studies are presented in the second chapter, which consists of a manuscript titled "Doppler shift compensation and audiogram in the low frequency horseshoe bat *Rhinolophus paradoxolophus* are comparable to that of other rhinolophids" that will be submitted soon for publication by the authors: Schoeppler, D., Kost, K., Schnitzler, H.-U. and Denzinger, A.

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Structure of the thesis

My thesis is a comparative echolocation study about two species belonging to the guild of flutter detecting foragers, with a large hipposiderid species, *Hipposideros armiger*, and an outlier rhinolophid species, *Rhinolophus paradoxolophus*. I structured this thesis in two chapters, the first chapter is already published in a peer-reviewed journal and the second chapter is prepared as manuscript for publication.

In the first chapter, I studied how the circadian variation of the body temperature affects the emission frequency of the Doppler shift compensating hipposiderid bat *Hipposideros armiger*. Implications for the audio-vocal control system of all high duty cycle bats are discussed. This chapter is already published: Schoeppler, D., Denzinger, A. and Schnitzler, H.-U. (2022). "The resting frequency of echolocation signals changes with body temperature in the hipposiderid bat *Hipposideros armiger*".

In the second chapter, I investigated the transmitter and receiver system of the outlier rhinolophid *Rhinolophus paradoxolophus*, which uses echolocation signals with an unexpectedly low CF_2. I investigated the Doppler shift compensation and echolocation behaviour and complemented these results with the measurement of the behavioural audiogram, which is the diploma thesis study of Katrin Kost. These results are the content of the second chapter with the title "Doppler shift compensation and audiogram in the low frequency horseshoe bat *Rhinolophus paradoxolophus* are comparable to that of other rhinolophids", which is prepared as manuscript by the authors: Schoeppler, D., Kost, K., Schnitzler, H.-U. and Denzinger, A.

Author information and contribution from others to chapter 1

The experiments were planned by me together with my advisers Annette Denziner and Hans-Ulrich Schnitzler, and I conducted the experiments, recorded, analysed and edited the data of chapter 1. I also wrote the first draft of the manuscript. The resulting paper was written in cooperation with my advisers and published with them as co-authors. Animals and technical resources were provided by Hans-Ulrich Schnitzler and Annette Denzinger.

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Chapter 1

The resting frequency of echolocation signals changes with body temperature in the hipposiderid bat *Hipposideros armiger*

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The resting frequency of echolocation signals changes with body temperature in the hipposiderid bat *Hipposideros armiger*

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Abstract

Doppler shift (DS) compensating bats adjust in flight the second harmonic of the constant-frequency component (CF_2) of their echolocation signals so that the frequency of the Doppler-shifted echoes returning from ahead is kept constant with high precision (0.1-0.2%) at the so-called reference frequency (f_{ref}). This feedback adjustment is mediated by an audio–vocal control system that correlates with a maximal activation of the foveal resonance area in the cochlea. Stationary bats adjust the average CF_2 with similar precision at the resting frequency (f_{rest}), which is slightly below the f_{ref}. Over a range of time periods (from minutes up to years), variations of the coupled f_{ref} and f_{rest} have been observed, and were attributed to age, social influences and behavioural situations in rhinolophids and hipposiderids, and to body temperature effects and flight activity in *Pteronotus parnellii*. We assume that, for all DS-compensating bats, a change in body temperature has a strong effect on the activation state of the foveal resonance area in the cochlea, which leads to a concomitant change in emission frequency. We tested our hypothesis in a hipposiderid bat, *Hipposideros armiger*, and measured how the circadian variation of body temperature at activation phases affected f_{rest}. With a miniature temperature logger, we recorded the skin temperature on the back of the bats simultaneously with echolocation signals produced. During warm-up from torpor, strong temperature increases were accompanied by an increase in f_{rest}, of up to 1.44 kHz. We discuss the implications of our results for the organization and function of the audio–vocal control systems of all DS-compensating bats.

KEY WORDS: Audio–vocal feedback control, Auditory fovea, CF-FM echolocation signals, Doppler shift compensation, Flutter detection

List of symbols and abbreviations

CF_2	constant-frequency component of the second harmonic
CRF	cochlear resonance frequency
DS	Doppler shift
f_{max}	frequency maximum
f_{ref}	reference frequency
f_{rest}	resting frequency
t_{max}	relative skin temperature maximum

Introduction

The auditory system of bats using the flutter detection echolocation strategy is highly specialized for the extraction of behaviourally relevant information in echoes from fluttering prey (for reviews, see Neuweiler et al., 1980; Schnitzler and Ostwald, 1983; Schnitzler and Denzinger, 2011; Fenton et al., 2012; Denzinger et al., 2018). Flutter-detecting foragers emit echolocation signals consisting of a long constant-frequency component, followed by a short downward frequency-modulated terminal part at high duty cycle, with highest amplitude in the second harmonic (CF_2). In flight, these bats use an extremely precise audio-vocal feedback control system to adjust the emission frequency so that the CF_2 from echoes, returning from stationary targets ahead, is kept constant at the so-called reference frequency (f_{ref}), which is defined as average CF_2 from all echoes (Schnitzler, 1968, 1973). By lowering the emission frequency, bats compensate for Doppler shifts (DS) in echoes from targets ahead over the whole range of their flight speeds (Schnitzler and Henson, 1980; Schoeppler et al., 2018). This echolocation behaviour has therefore been termed DS compensation (Schnitzler, 1967), and is found in rhinolophid (Schnitzler, 1968) and hipposiderid bats (Gustafson and Schnitzler, 1979) and in the mormoopid bat *Pteronotus parnellii* (Schnitzler, 1970). In playback experiments, stationary horseshoe bats also compensated for simulated positive DS and adjusted CF_2 of the playback echoes at f_{ref} (Schuller et al., 1974), although negative shifts were not compensated for.

DS-compensating bats possess an auditory fovea in the cochlea, consisting of a morphologically specialized resonance area with a disproportionally high overrepresentation of hair cells tuned to frequencies at or near f_{ref} (Suga et al., 1975; Bruns, 1976; Schuller and Pollak, 1979; Kössl and Vater, 1985; for reviews, see Kössl and

Vater, 1995; Vater, 2004). By the adjustment of echo CF_2 at f_{ref}, DS compensation leads to a maximal activation of the resonance system in the inner ear. Afferent connections from the auditory fovea in the cochlea project to foveal areas in higher centres of the entire ascending auditory pathway with an overrepresentation of many sharply tuned neurons with best frequencies at or near f_{ref} (reviewed in Neuweiler et al., 1980; Suga, 1984; Schnitzler and Denzinger, 2011). The auditory fovea is also featured in the auditory threshold, where the threshold minimum is narrowly tuned to the f_{ref} with a distinct threshold increase to lower frequencies (reviewed in Schnitzler and Denzinger, 2011). The sensory information encoded in these foveal areas acts on motor areas for vocal control and determines the CF_2 of emitted echolocation signals, thus completing the audio-vocal control loop (Schuller and Rübsamen, 1981; Rübsamen and Betz, 1986; Metzner, 1989; Kössl and Vater, 2000).

When flutter-detecting bats vocalize while resting, they keep the CF_2 of their signals similarly constant with high precision at the so-called resting frequency (f_{rest}; CF_2 average of emitted pulses) with deviations of not more than 0.1–0.2% (reviewed in Schnitzler and Denzinger, 2011; Schoeppler et al., 2018). In all flutter-detecting foragers, f_{ref} and f_{rest} are tightly coupled with a species-specific offset between the two of not more than a few hundred Hz (reviewed in Schnitzler and Denzinger, 2011). This constancy of f_{rest} and f_{ref} has been supported in experiments over only very short time periods (i.e. a few seconds). At longer time periods, however, intra-individual variations of f_{rest} have been documented for several species of flutter-detecting foragers (rhinolophids: Long and Schnitzler, 1975; Hiryu et al., 2008; Furusawa et al., 2012; hipposiderids: Riquimaroux, 2000; Hiryu et al., 2006; Schoeppler et al., 2018; *P. parnellii*: Suga et al., 1987; Gaioni et al., 1990). A change of f_{rest} over the course of several days or months often occurred as a gradual decrease with time, sometimes accompanied by reversion back to the original f_{rest}. The highest individual variations have been observed in hipposiderids, in a long-term study of *Hipposideros terasensis*, all bats decreased or increased their f_{rest} in the same direction with variations of 3 kHz on average (Hiryu et al., 2006). Integration of new individuals to a preexisting colony usually provoked an increase in frequency and isolations and always resulted in large drops in f_{rest}. A common feature of all these reports is that the documented intra-individual frequency shifts relate to specific behavioural situations and/or to a specific social context (Hiryu et al., 2006) but do not offer a physiological mechanism which explains them.

Physiological mechanisms which may be responsible for variations of f_{rest} and f_{ref} have only been studied in the mormoopid bat *P. p. parnellii* (Henson et al., 1990; Huffman and Henson, 1991, 1993a,b). These studies found that changes of the cochlear resonance frequency (CRF), which were induced by flight activity, body temperature and contralateral noise, resulted in variations of the f_{rest} and the coupled f_{ref} in bats sitting on a swinging pendulum (Huffman and Henson, 1993a). These changes also had an effect on the best frequencies of neurons in the cochlear nucleus and in the inferior colliculus (Huffman and Henson, 1991; 1993b). The measured effects of body temperature reported in these experiments were somewhat contrasting. In the first study (Henson et al., 1990) the CF_2 changes did not consistently correlate with temperature changes induced by flight activity. However, in the following experiments (Huffman and Henson, 1991, 1993a,b), a clear temperature dependency was demonstrated when the animals were warmed up by a heat lamp.

These studies on the mormoopid bat suggest that f_{rest} and f_{ref} might be labile and dependent on the physiological state of a bat. Results suggest that the temperature-induced frequency shifts result from changes of the resonance properties of the foveal area in the cochlea. The closely related rhinolophid and hipposiderid bats also compensate for DS, but they are phylogenetically more distant from *P. parnellii* (Jones and Teeling, 2006). Their auditory fovea is also based on specific resonance properties of the cochlea but differs in its morphology and physiology from that of *P. parnellii* (Henson et al., 1985; Kössl, 1994; for reviews, see Vater, 2004; Neuweiler, 2003). Hence, mechanisms which induce changes in resonance properties in *P. parnellii* are not necessarily those that cause the changes in rhinolophids and hipposiderids.

The aim of this study is to understand how a physiological parameter (body temperature) affects f_{rest} of the echolocation signals in the hipposiderid bat *Hipposideros armiger*. We hypothesized that the activation of the foveal resonance system in the cochlea of this species (and presumably of other rhinolophid and hipposiderid bats) will change with body temperature and should lead to a concomitant frequency change of the emitted echolocation signals. With an external miniature temperature logger placed between the scapulae, we measured the diurnal change of skin temperature continuously over periods of 34 h, recorded simultaneously the echolocation signals of vocalizing bats, determined their resting frequency, and correlated f_{rest} with the measured skin temperature. We discuss our results with regard to our current view on the organization of the audio-vocal control system for DS compensation, and in view of the resistance of DS compensation to disturbances by temperature effects.

Materials and methods

Animals and ethical statements

Experiments were conducted with two adult female Vietnamese round-leaf nosed bats, *Hipposideros armiger* (Hodgson 1835), captured 2009 in the Ba Be National Park in Vietnam with permission from 13 May 2009 granted to the Vietnamese Institute of Ecology and Biological Resources, Hanoi (no. 129/STTNSV). Bats were kept at the animal facility of the Institute for Neurobiology of the University of Tübingen in a room (6×3.6×3 m) under controlled abiotic conditions (12 h:12 h light:dark cycle, 26.6±2°C and 60±5% humidity) and housed separately in two aviaries (2.4×1.2×2 m and 3.2×1.25×2 m). During the non-experimental time the aviaries were open so that the bats could freely fly in the room. Water and mealworms (larvae of *Tenebrio molitor*) were available *ad libitum* and semi-monthly vitamin and mineral supplements (Nutri-Cal® Albrecht GmbH, Germany; Efaderm® aristavet GmbH & Co., Germany; Korvimin® WDT eG, Germany) were administrated. Additionally, crickets (*Gryllus* spp., *Acheta domestica*), locusts (*Schistocerca gregaria*) and beetles (*Zophobas morio*, *Pachnoda marginata*) were hand-fed to the bats.

During this time, bats were trained to carry a miniature temperature logger on their back. Using positive reinforcement with favoured food like beetles and locusts, the bats learned to climb on the hand of the experimenter. When bats were accustomed to handling, we shaved off a small patch of fur between the shoulder blades and fixed a dummy with water-soluble glue (Mastix watersoluble, GRIMAS® B.V., Holland; residue-free removable, safe for use with children) onto the shaved spot between the shoulder blades to get the bat accustomed to an object. After this habituation procedure, we attached the miniature temperature logger for registration of skin temperature. The mass of the logger was 1.66 g and corresponded to less than 3% of subject body mass, which was 60 g on average. The logger did not affect behaviour. After each session, the logger was removed with water.

The experiments were conducted in accordance with the German Animal Welfare Legislation and did not require explicit approval (letter from the approval authority from 29 March 2012). The license to keep *Hipposideros armiger* was issued by the responsible agency (Regierungspräsidium Tübingen, Germany).

Experimental setup and recordings

During each experiment we recorded the skin temperature between the shoulder blades every minute with an i-Button ETL1 miniature temperature logger (Maxim Integrated, USA). The logger had a temperature range from +15 to +46°C and a resolution of 0.125°C. During this time, we also made continuous sound recordings (PCtape, Animal Physiology, University of Tübingen). The echolocation signals were picked up with a custom-made ultrasonic microphone (frequency response within 5 dB in the range of the echolocation signals), amplified, digitized with a sampling rate of 480 kHz, 16-bit, and stored as .wav files. The microphone was positioned around 80 cm under the bat's preferred resting place and recorded all emitted signals with sufficient quality independent of the bat's position in the aviary.

Each bat was tested in two separate sessions each lasting 34 h. The recordings started approximately 5 h before the lights were turned off. The experimenter entered the room 1-2 h after the beginning of this dark phase and stayed in the room for up to 25 minutes conducting usual husbandry activities. Additionally, the experimenter entered the room again at the end of the dark phase and checked visually the condition of the animal and the fit of the logger. During the experiment phase, bats had been familiar both with this procedure and the experimenter for several months prior. After warming up, bats generally flew to the experimenter and landed nearby or even on the hand to receive crickets or beetles.

Data analysis and statistics

Data from the temperature logger were exported via an USB reader and OneWireViewer software (both Maxim Integrated). Depending on the fit of the logger, the maximum skin temperature varied up to 3°C between the sessions. Therefore, we calculated the skin temperature relative to the maximum of one session (Figs 1 and 2) and relative to the maximum of each rise (Figs 3B, 4 and 5; Fig. S1). Temperature values were not normally distributed (Shapiro Wilk test $P<0.05$). Therefore, a Wilcoxon rank sum tests was conducted, to test for differences in skin temperature between light and dark phases with two complete 24 h cycles lasting from 08:00 to 07:59 h per bat.

Sound analysis was conducted with the software Selena (University of Tübingen) in colour spectrograms. We first measured the calling activity of a bat over 24 h to test whether the calling activity was associated with change in skin temperature. To determine calling activity we subdivided the 24 h recording in time bins of 3 min. For every time bin, we determined in steps of 5 s whether the bat was vocalizing. We defined calling activity if the bat emitted at least 10 signals within the 5 s time slot. The calling activity within the 3 min time bin was calculated as the percentage of calling activity from the thirty-six 5 s time slots of each 3 min bin. For example, the calling activity in the 3 min time bin was 100% if the bat emitted at least 10 calls during each 5 s interval. Because a normal distribution was not observed across the data (Shapiro-Wilk test $P<0.05$), we performed a Wilcoxon rank sum test to test whether the calling activity differed between the light and dark phase.

To measure changes in CF_2 caused by the activation of the bats, we determined the CF_2 of resting signals, also known as resting frequency (f_{rest}), emitted 2 h before and 2 h after the entrance of the experimenter. Calls were displayed in a 5 s window as spectrograms between 60 and 75 kHz using a fast Fourier transform with 8192 points (zero padding), which resulted in a frequency resolution of 20.25 Hz. f_{rest} was manually measured as average maximum frequency over all calls in the 5 s window. We only used 5 s time slots with at least 10 signals as single calls, and in some cases the first few calls (up to three) in slots with calling activity were very different in frequency compared to the others. They were excluded from f_{rest} measurements.

Further, we correlated the increase in skin temperature from beginning of activation to its maximum with the percentage calling activity in the 3 min time span before the entrance of the experimenter, and tested it using a Spearman correlation. Although the Shapiro-Wilk test ($P>0.05$) indicated a normal distribution, we conducted a nonparametric test to reduce the impact of outliers. To correlate the increase in skin temperature with the increase of f_{rest} from activation to its maximum, we averaged the f_{rest} per minute. We tested for significance with a Spearman correlation (normal distribution was not observed, Shapiro-Wilk test $P<0.05$).

We plotted the normalized f_{rest} and the normalized skin temperature over time from the point of entrance to the respective maximum. Additionally, we determined the minimum in skin temperature from the peak in skin temperature within 30 minutes thereafter. We fitted the correlation with linear trend lines to the increasing part after activation until frequency maximum, and to the decreasing part from skin temperature maximum until its described minimum. The slopes of these trend lines were statistically tested with the sign test (Sokal and Rohlf, 1995).

Results

Diurnal rhythm of skin temperature

In two individuals of *H. armiger* (referred to hereafter as “bat 1” and “bat 2”), skin temperature was continuously measured every minute in two sessions, each lasting 34 h. Skin temperature was never stable. However, the diurnal pattern of the change in skin temperature was rather similar between both session and animal, and indicated the influence of the light:dark cycle as well as the influence of external stimuli, e.g. the entrance of the experimenter (Fig. 1). Skin temperature was slightly higher during dark phase, when the nocturnal bats were more active than during the light phase. Relative to the normalized maximum skin temperature (tmax) reached in a given session, the median of the skin temperature was 2.1°C (bat 1) and 2.8°C (bat 2) below tmax in the dark phase and 2.9°C (bat 1) and 4.3°C (bat 2) below tmax in the light phase. (bat 1: $Z=-10.35$, $n_{light}=1440$, $n_{dark}=1440$, $P<0.0001$, bat 2: $Z=-23.26$, $n_{light}=1440$, $n_{dark}=1440$, $P<0.0001$). The highest difference to tmax was measured during the light phase with relative values of 4.9 to 5.9 °C below tmax. The strongest effects of distinct skin temperature rises were observed when the experimenter entered the room. After this external stimulus the tmax was reached within 4–21 min (Table 1) and deceased afterwards at a slower rate. The relative increase in skin temperature depended on the skin temperature prior to stimulation and was higher at initially low skin temperatures

(Fig. 1). For further analysis of temperature effects on f_{rest} we concentrated only on the distinct skin temperature increases resulting from the activation of the bats by the experimenter (see Fig. 1).

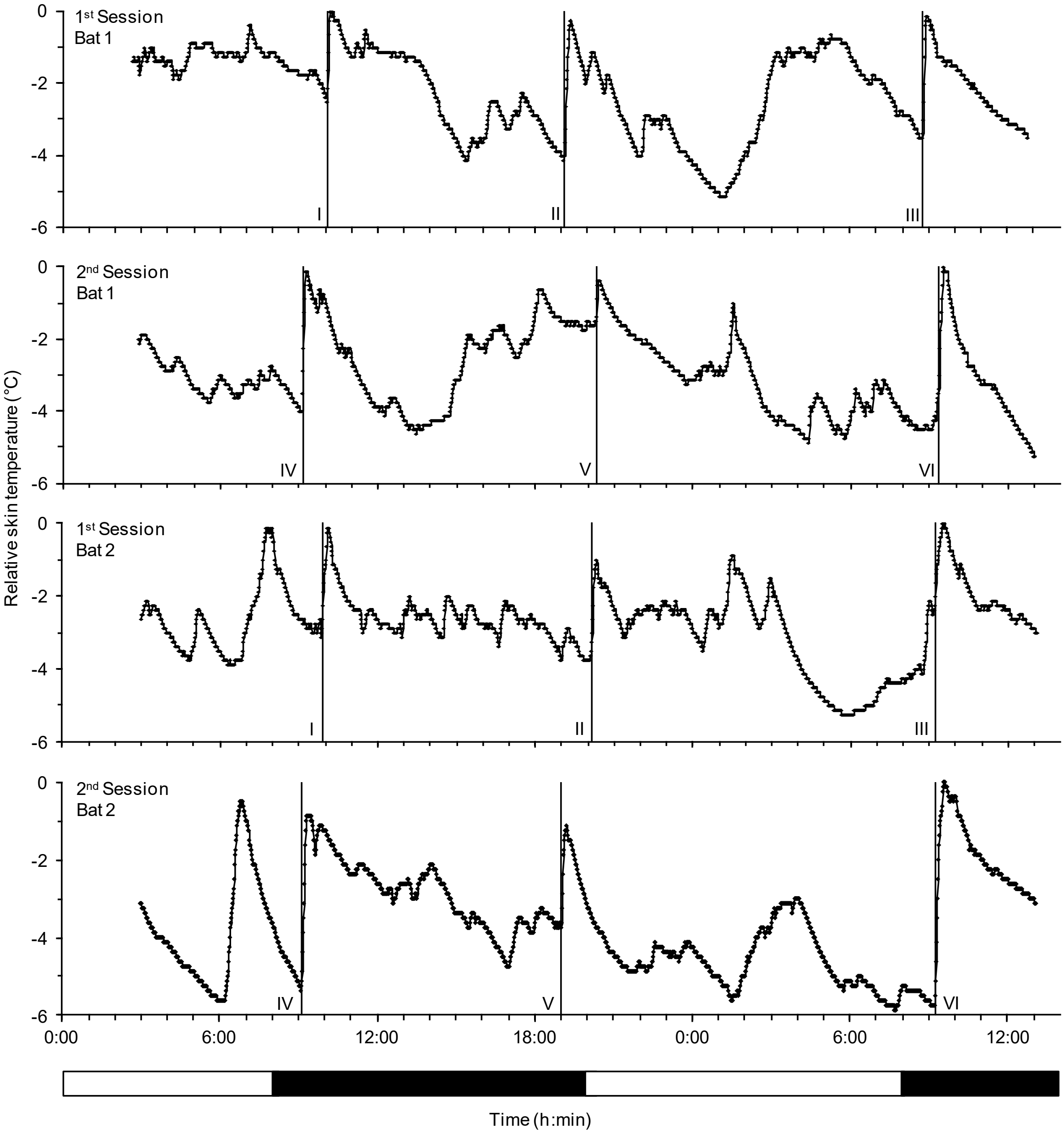

Fig. 1. Relative skin temperature of bat 1 and bat 2. The temperature was measured every minute over a period of 34 h in two sessions per bat. Skin temperature is shown relative to the maximum during each period. Bats increased body temperature when the experimenter entered the room which is marked by a vertical line (no. I–VI). The bar below indicates the light:dark cycle (12 h:12 h). The light was turned off at 08:00 h and turned on at 20:00 h.

Variation of skin temperature and calling activity

The diurnal calling activity pattern is characterized by periods lasting up to 26 min where bats emitted more or less continuous calls separated by longer silent periods (Fig. 2A). Calling activity was influenced by the light:dark cycle as well as by external stimuli (Fig. 2). Overall, average calling activity during light periods (average 9%) did not significantly differ from calling activity during dark periods (average 12%, Z=-0.3, n_{light}=241, n_{dark}=242, *P*=0.7637). Calling activity was high at rising skin temperatures (Fig. 2B,D). The minimum in skin temperature occurred during light periods, and in the example in Fig. 2B it was correlated with the lowest calling activity (silence for 2 h). The strongest effects, with a distinct increase in skin temperature and in calling activity, were observed when the experimenter entered the room (Fig. 2; Fig. S1). Therefore, we used this reaction to investigate the relation between f_{rest} and skin temperature. The bat displayed in Fig. 2C,D increased calling activity approximately 4 min before the experimenter entered the room, followed by an increase in skin temperature. Calling activity remained at 100% as long as the experimenter was in the room and dropped rapidly after experimenter exit, accompanied by a slight decrease in skin temperature (Fig. 2C,D).

Table 1. Skin temperature and resting frequency for each bat at all activations by the experimenter (I-VI)

			Skin temperature				Resting frequency		
	No.	Calling activity before activation %	t_{max} (°C)	Δt (°C)	Δtime(t_{max}) (min)	t at f_{max} (°C)	f_{max} (kHz)	Δf (kHz)	Δtime(f_{max}) (min)
Bat 1	I	42	32.13	2.50	7	31.50	65.81	0.40	5
	II	0	31.88	3.75	14	31.13	66.11	0.92	9
	III	0	32.00	3.38	10	31.38	65.76	0.78	7
	IV	6	34.63	3.88	9	33.13	65.77	0.49	5
	V	19	34.38	1.13	4	34.38	65.22	0.39	4
	VI	19	34.75	3.50	11	33.25	65.87	0.32	5
Bat 2	I	17	32.88	2.88	13	31.63	66.03	0.45	6
	II	22	32.00	2.63	12	31.25	66.35	0.42	6
	III	14	33.00	2.38	18	31.63	65.83	0.24	4
	IV	8	32.75	4.50	13	31.50	66.19	1.44	9
	V	19	32.50	2.63	12	30.38	65.76	0.25	2
	VI	0	33.63	5.75	21	31.63	66.03	1.25	9

t_{max}, maximum skin temperature; f_{max}, maximum resting frequency; Δt and Δf, increase of skin temperature and frequency from the instance when the experimenter entered and the corresponding maximum; Δtime(f_{max}) and Δtime(t_{max}), the duration to reach maximum values; t at f_{max}, the skin temperature at the frequency maximum. Calling activity was measured during a 3 min period directly before activation.

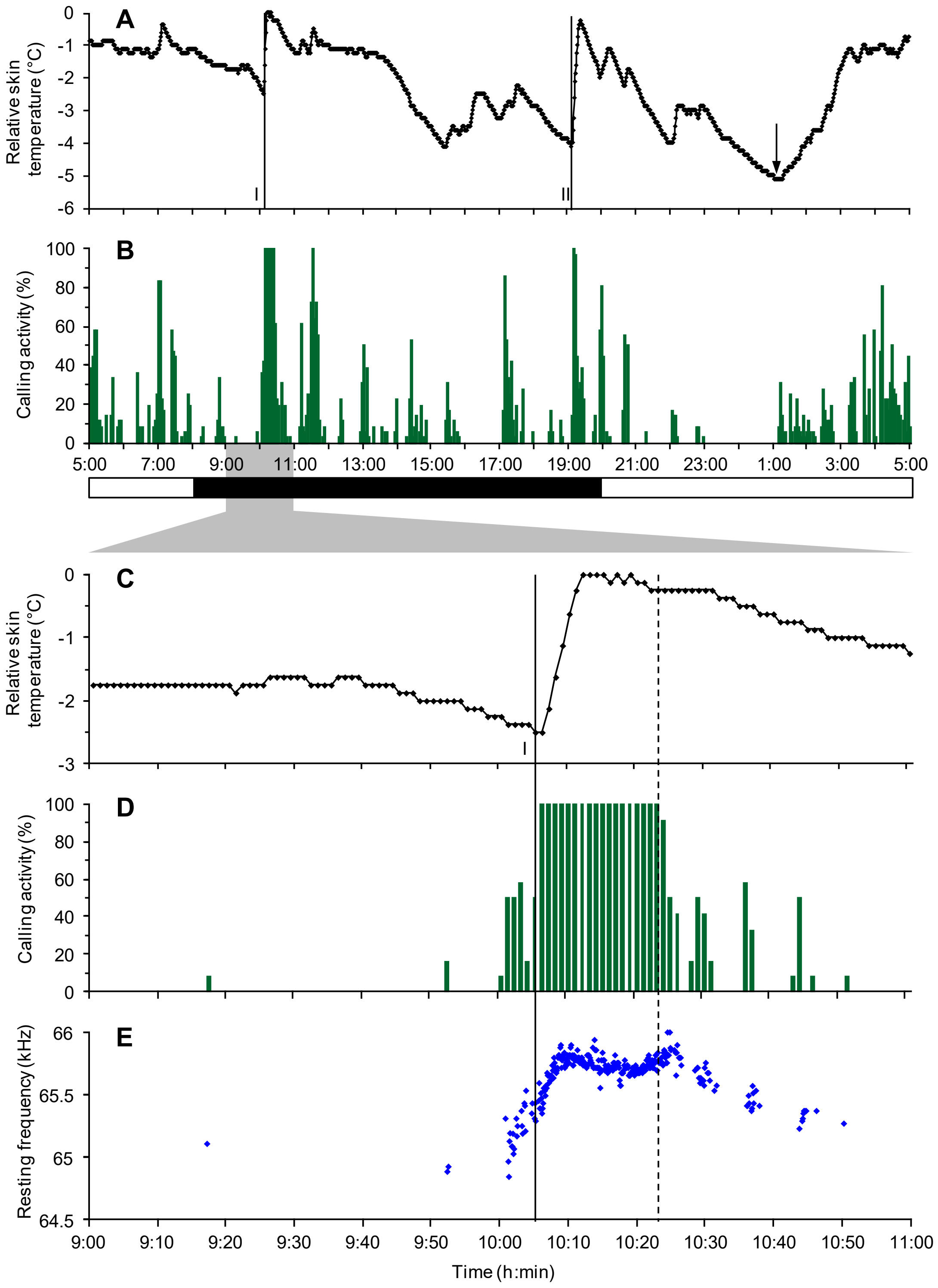

Fig. 2. Echolocation signal emission and skin temperature course over 24 h and one activation in detail. Relative skin temperature (A,C), calling activity (B,D) and resting frequency (E) of one bat during a 24 h period (A,B) and the 2 h period (C–E) around the first activation (I) by the experimenter (black line). Skin temperature is shown relative to the daily maximum (32.1°C). The minimum is marked with an arrow (A) and occurred at the end of the longest silent period. Calling activity was depicted in time bins of 3 min (B) and 1 min (D). Each frequency value in E represents the resting

frequency in a 5 s slot. The vertical lines in C–E mark the instant of time when the experimenter entered (solid line) and left (dotted line) the husbandry room.

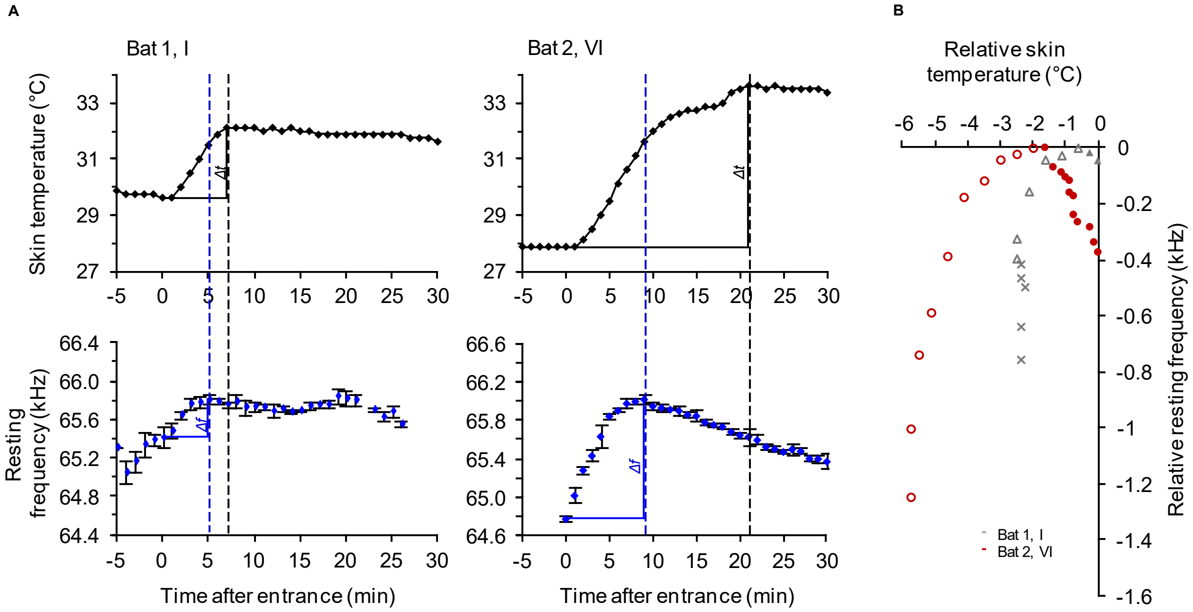

Fig. 3. Exemplary course of skin temperature and resting frequency during activation, with and without calling activity before. (A) Absolute skin temperature and resting frequency in the time periods 5 min before and 30 min after activation by the experimenter (no. I of bat 1 and no. IV of bat 2; see Fig. S1). (B) Correlation between the same data normalized to their maximum values. Each dot corresponds to a 1 min period, the resting frequency was averaged (mean±s.d.). In A, the dotted blue lines indicate the frequency maxima and the dotted black lines the skin temperature maxima. Solid lines mark the temperature and frequency rise from activation to the maximum. In B, × symbols depict values 5 min before activation, open symbols depict the values from activation until the frequency maximum is reached and filled symbols depict the values to the temperature maximum after the frequency maximum was reached.

Variation of skin temperature and resting frequency

When activated by the experimenter, *H. armiger* reacted not only with an increase in skin temperature and calling activity, but also shifted the CF_2 of the resting signals (f_{rest}) continuously towards higher frequencies (Figs 2E, 3A; Fig. S1). Both skin temperature and f_{rest} increased steeply to a maximum (Figs 2E, 3A, Table 1; Fig. S1). Generally, bats with a high calling activity in the 3 min prior to activation also had a higher initial skin temperature and a higher initial f_{rest}, and therefore a smaller increase in each (Table 1, Figs 3A and 4A). Increase in skin temperature and calling activity were inversely correlated (Spearman's ρ =-0.675, N=12, P=0.0160) (Fig. 4A). The maximum in f_{rest} (f_{max}) was reached earlier (within 2–9 min) than the t_{max} (within 4–21 min), except in one case, when the frequency and the skin temperature simultaneously reached the maximum after 4 min (Table 1). The relative increase of the f_{rest} ranged between 0.24 and 1.44 kHz with increase rates of 60-160 Hz min^{-1} or 90–350 Hz °C^{-1} (skin temperature). The t_{max} was reached on average 6±4 min after the f_{max}, but the longest interval measured 14 min. The relative increase of skin temperature ranged between 1.13 and 5.75°C, with increase rates between 0.13 and 0.43°C min^{-1}. The relative increases in frequency were positively correlated with relative increases in skin temperature (Spearman's ρ=0.79, N=12, P=0.0022) (Fig. 4B).

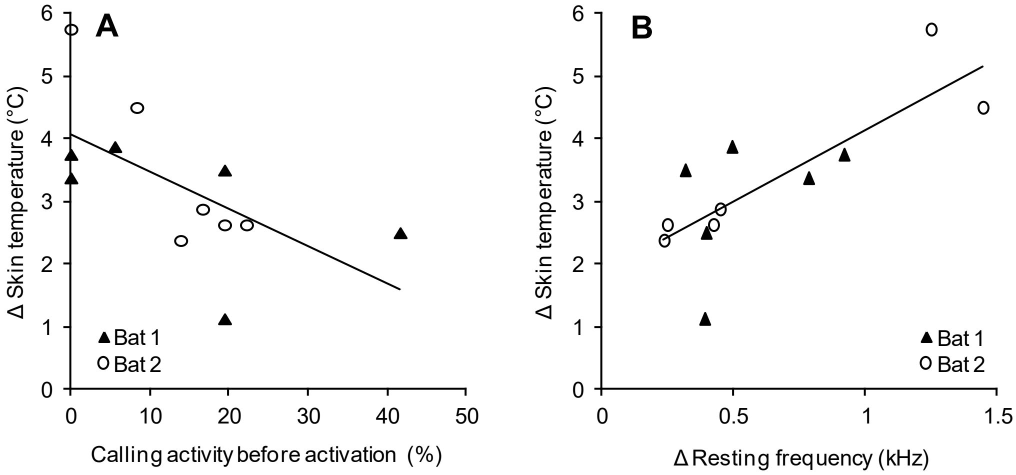

Fig. 4. Relationship between skin temperature and echolocation signal emission. (A) Correlation between the increase in skin temperature and calling activity before activation and (B) the increase in resting frequency and increase in skin temperature for all six activations in the two bats with regression lines. The calling activity before activation (A) is based on the time span 3 min before the experimenter entered the room. (A) Spearman's ρ=−0.675, N=12, P=0.0160; (B) Spearman's ρ=0.79, N=12, P=0.0022.

In the time span between f_{max} and t_{max}, the skin temperature increased by 1.1°C on average (0.6-2.1°C) in 11 of 12 cases, whereas the f_{rest} was decreasing slightly, by an average of 70 Hz. Only in activations IV and VI of bat 2 (Figs 3B and 5) was the f_{rest} reduction much higher (values of 320 and 390 Hz, respectively; Table 1, Figs 3 and 5). After the t_{max} was reached, skin temperature and f_{rest} decreased slowly in both bats (Fig. 5; Fig. S1). Skin temperature dropped at an average rate of 0.04°C min^{-1} and reached a minimum within 18–30 min (0.75-1.5°C below the maximum). f_{rest} decreased to the range of 90–600 Hz, with an average rate of 10 Hz min^{-1} or 390±240 Hz °C^{-1} (skin temperature), and 310±160 Hz °C^{-1} (skin temperature) after removal of two bat 2 outliers (IV and VI). Although the peaks of these two variables did not occur at the same time, the f_{rest} always increased with increasing skin temperature until f_{max} was reached (sign test P=0.031 for each bat). Likewise, the f_{rest} decreased concurrently with decreasing skin temperature after the t_{max} was reached (sign test P=0.031 for each bat) (Fig. 5).

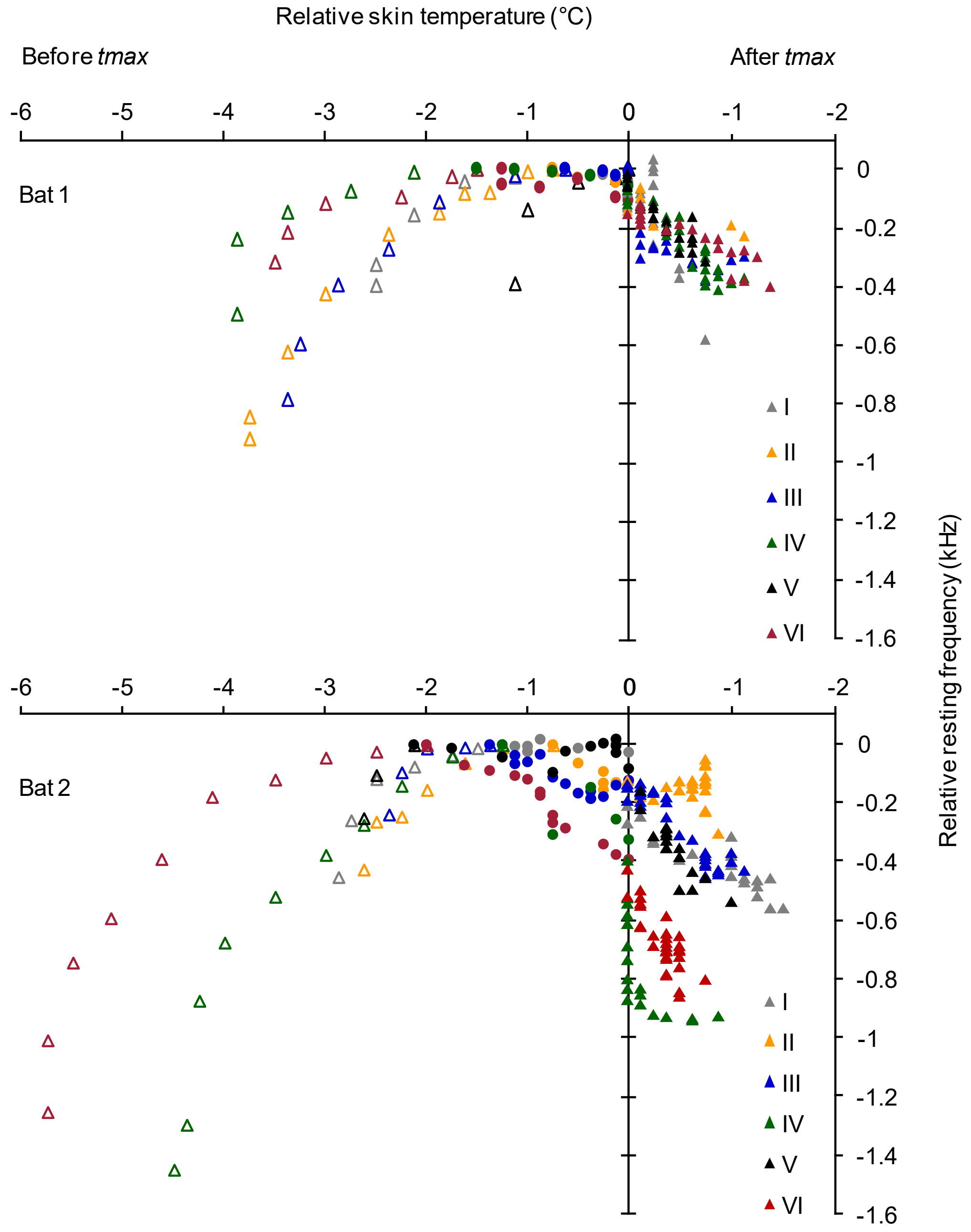

Fig. 5. Correlation between relative resting frequency and the relative skin temperature of bat 1 and bat 2 for all activations (I–VI). Each curve presents data from activation to the frequency peak (open symbols), between frequency and temperature peak (filled circles), and data after the temperature peak (filled triangles). The latter is given on a second negative *x*-axis to the right for better separation of data points. Each dot corresponds to the average value of a 1 min period. Per bat (*N*=6) sign test *P*=0.031.

Discussion

In this study, we investigated the influence of the circadian changes of body temperature on the CF_2 of resting signals (resting frequency or f_{rest}) in *H. armiger.* With this approach, we tested the hypothesis that body temperature influences the resonance conditions in the foveal area of the cochlea in a DS-compensating hipposiderid bat. Previous work had suggested this occurs in a mormoopid bat (Huffman and Henson, 1991, 1993a,b), but this had never been tested in hipposiderid or rhinolophid bats before. The concomitant change in cochlear output should lead to a different activation of the audio-vocal control system, which should result in a readjustment of the emission frequency and its rise with increasing body temperature.

The circadian variation of the skin temperature of *H. armiger* was strongly determined by external Zeitgeber (Fig. 1). At the beginning and end of a dark phase, bats reacted to the entrance of the experimenter with a distinct increase of body temperature to a maximal value, which was always accompanied by a continuous emission of signals with rising f_{rest} (Fig. 2; Fig. S1). Sometimes bats increased their calling activity prior to experimenter entrance, which may indicate that they were accustomed to the regular husbandry procedures and were waiting to be fed. The magnitude of the induced skin temperature shifts varied strongly, for instance, between 1.13°C (bat 1/V) and 5.75°C (bat 2/VI). Skin temperature shifts of similar size were also observed in other parts of the dark phases and even during some light phases. However, often they were not accompanied with a continuous calling activity. Therefore, we concentrated analyses only on activation periods, which were induced by the experimenter.

After the bats' activation, values of both skin temperature and f_{rest} increased to a maximum. Generally, frequency maxima (f_{max}) were reached earlier than skin temperature maxima (t_{max}). In the time span between f_{max} and t_{max}, the skin temperature continued to increase and afterwards was maintained at the t_{max} level for some time before it was slowly reduced.

If it is indeed temperature that is determining f_{rest}, the question remains as to why f_{max} was reached before the t_{max} of skin temperature. One explanation could be that the skin temperature, which we measured with our sensor between the shoulder blades, differed from the temperature in the cochlea, the location that is hypothesized to affect the adjustment of f_{rest}. Several studies suggest that at constant ambient temperature, the skin temperature, measured in a neck fold or between the scapulae, gives a good approximation of body temperature (e.g. Audet and Thomas, 1996; Barclay et al., 1996; Henson et al., 1990; Huffman and Henson, 1993a; Willis and Brigham, 2003). However, during the warming-up phase of torpid bats, Willis and Brigham (2003) observed differences between the core temperature of body and skin temperature. Body temperature measured continuously with an implanted transmitter in the intraperitoneal cavity and compared to the skin temperature between the scapulae suggested that upon arousal from torpor, the increase rate in body temperature (0.52 °C min^{-1}) was higher than the increase rate in skin temperature (0.44 °C min^{-1}). This may explain why the body core temperature along with cochlear temperature reached their maxima earlier than skin temperature. Owing to the fact that the measured increase in skin temperature does not exactly reflect the increase of the core temperature and/or cochlea temperature, we cannot determine the average increase rate of frequency relative to body temperature.

At 10 of the 12 activations (except activations IV and VI of bat 2), a moderate increase in skin temperature led to the f_{max}, which was reached between -2°C and the t_{max} and was maintained until the relative skin temperature had reached its maximum. Afterwards, the body temperature and f_{rest} slowly decreased, which was also accompanied by a decrease in skin temperature. The exceptions are activations IV and VI of bat 2, where the overall increase in skin temperature was higher. These two activations have in common that the skin temperature at beginning of the activation was distinctly lower, which indicates that the bat may have been in a state of deeper torpor in these situations, and which may explain these differing results.

From these experiments, we conclude that in *H. armiger* body temperature determines CF_2 of the emitted signals, and that an increase in temperature leads to an increase in emission frequency. According to the close phylogenetic relationship, we propose that this result may also hold for all hipposiderid and rhinolophid bats, although further testing of this hypothesis is warranted.

The influence of body temperature on the CF_2 in a DS-compensating bat was only previously shown in the mormoopid *P. parnellii* (Huffman and Henson, 1993a). That study found that changes of body temperature correlated positively with the CF_2 of the coupled f_{rest} and f_{ref} and also with the CRF. Further, flight and contralateral sound exposure also shifted CRF, and along with it the f_{rest} and f_{ref}. Temperature also had influence on the offset between f_{rest} and f_{ref}, which was reduced slightly with increasing temperature. The authors concluded that the induced changes of CRF were responsible for the observed changes of CF_2 in stationary bats and in DS-compensating bats on a pendulum. They discussed the possible existence of a feedback mechanism for sound emission, where a ‘set point’ of neural activity in the vocal centres detects the coincidence of both the activation of the narrow band to which the cochlea is most sensitive, and the frequency of the emitted CF_2. Huffman and Henson (1991, 1993b) complemented the temperature studies in *P. parnellii* by showing that the neuronal tuning of foveal neurons of the cochlear nucleus and of the inferior colliculus were also labile, and could change with temperature.

Further evidence that the tuning of the cochlea of DS-compensating bats depends on their physiological state comes from studies with anesthetized bats. Audiograms of anaesthetized rhinolophids and hipposiderids differ conspicuously from those of non-anesthetized bats, similar to observations of *P. parnellii* [Neuweiler, 1970: *Rhinolophus ferrumequinum*; Pollack et al., 1972: *Chilonycteris p. parnellii* (*P. parnellii*); Foeller and Kössl, 2000: *H. lankadiva*). Further, in a study in which the physiological properties of the inner ear of the rhinolophid *Rhinolophus rouxii* and the mormoopid *P. parnellii* were directly compared, Henson et al. (1985) not only described the influence of anaesthesia on the sensitivity and tuning of audiograms in *R. rouxii*, but also suggested (without reporting corresponding data) that the cochlear microphonic audiograms were affected by temperature. Effects of temperature and anaesthesia on the foveal resonance system of *P. parnellii* were also reported from Kössl and Vater (1985), who measured cochlear microphonic potentials and evoked otoacoustic emissions.

All DS-compensating bats possess a mechanical resonance system, which is the basis for the auditory fovea, a highly expanded frequency representation in the range of CF_2. Therefore, we propose similar influences on the foveal resonance system in the cochlea and concomitant shifts in CF_2, regardless of the phylogenetic relationship of taxa. While acknowledging the marked differences in cochlear mechanics of the foveal tuning system in *P. parnellii* and in rhinolophid and hipposiderid bats (Henson et al., 1985), we assume that similarities in the effects of temperature and anaesthesia on the foveal tuning and the concomitant adjustment of CF_2 as demonstrated in the current study for *H. armiger* and by others for *P. parnellii* indicate a similar audio-vocal control principle in all DS-compensating bats.

We found that the variation of body temperature of *H. armiger* results in a concomitant change in f_{rest}. Owing to the coupling of f_{rest} and f_{ref}, we are confident that temperature effects both frequencies in a similar way. The temperature-dependent variability of the CF_2 allows conclusions on the function of the audio-vocal control system of DS-compensating bats. The controlled process variable of the audio-vocal feedback control system is the activation state of the foveal resonance area in the cochlea. This cochleotopic activation status is reported to the audio-vocal control centre by the afferent foveal areas of the auditory pathway. The emission frequency is changed if the reported process variable differs from the set-point condition of the central control system. At deviations, some kind of push/pull mechanism changes via efferent motor pathways the emitted CF_2, with inhibitory feedback lowering and excitatory feedback increasing the emitted CF_2 (Metzner et al., 2002). A change of the emitted CF_2 modifies, through feedback, the whole auditory input consisting of the emitted signal and of all returning echoes until the foveal input into the vocal control centre has reached the set-point condition again. Flying bats perceive the auditory input consisting of the emitted signal and its delayed DS echoes and adjust the emission frequency in such a way that the highest echo frequency in the perceived pulse-echo train is, independent of flight speed, kept constant at the so-call reference frequency (average of echoes with highest DS returning from ahead) with a standard deviation of 0.1–0.2% (Schnitzler and Denzinger, 2011; Schoeppler et al., 2018). Resting bats perceive the auditory input consisting of the emitted resting signals and their delayed non-DS echoes and adjust the emission frequency at the so-called resting frequency (averaged emission frequency of stationary bats), which is kept constant within short periods (again with a standard deviation of 0.1–0.2%, Schnitzler and Denzinger, 2011; Schoeppler et al., 2018). The coupling between resting and reference frequency indicates that the activation state of the cochlea by the pulse-echo train of resting and of reference frequency is most likely similar if the two coupled frequencies are separated by the observed offset, between 50 and 300 Hz (Schnitzler and Denzinger, 2011). Our data from *H. armiger* and previous data from *P. parnellii* (Huffman and Henson, 1991, 1993b) suggest a similar mechanism of the audio-vocal control system in all other DS-compensating hipposiderids and rhinolophids.

We suggest here that the tuning of the hard-wired auditory fovea cannot be deliberately varied. According to the data collected, all reported changes of resting and/or reference frequency in DC-compensating bats originate from the audio-vocal control system either by morphological or physiological changes of the resonance system. Irreversible changes of CF_2 in adult bats may be related to aging processes, which change the tuning properties of the foveal resonance system. The observation that wild living *R. ferrumequinum* aged 10–23 years (Jones and Ransome, 1993) dropped f_{rest} by about 200 Hz $year^{-1}$ and that one individual of *H. armiger* and one individual of *Rhinolophus paradoxolophus* dropped CF_2 by 1 and 0.9 kHz, respectively (D.S., personal observation) may be explained by age-related morphological changes in the inner ear. A well-studied example of how growth-related changes in morphology influence the CF_2 is the increase of CF_2 of young DS-compensating bats during ontogeny (for reviews, see Rübsamen, 1992; Vater et al., 2003).

We assume, that, if aging processes can be excluded, variations in f_{rest} and/or the coupled f_{ref} underlie reversible physiological mechanisms that influence the nature of the travelling wave. It has been shown that cochlear micromechanics changed with temperature. For instance, weak temperature effects were found in the motility of the outer hair cells of guinea pigs (reviewed in Ashmoore, 2008). Though these effects seem to be marginal in other mammals, they may be distinctive in DS-compensating bats owing to the high expansion of the frequency representation at their auditory fovea.

For instance, changes of f_{rest} and/or f_{ref}, which have been attributed to social interactions, may in fact be related to temperature effects (Hiryu et al., 2006; Furusawa et al., 2012). A strong support for our temperature hypothesis is the observation that a drop in body temperature was indicative of a subject that was close to death. Furusawa et al. (2012) described a large frequency drop of 2 kHz in a sick bat before it perished. Also, Hiryu et al. (2006) measured a significant decrease in f_{rest} before a bat died. We suggest that these sick bats reduced their body temperature and with it f_{rest}. In future studies on the effects of social interactions on CF_2, it should also be tested whether these interactions occur simultaneously with body temperature changes, which could also explain the observed frequency changes.

Besides DS-compensating bats, other vertebrates also have hearing systems that are influenced by body temperature. Changes in the sensitivity of audiograms and of also the tuning of single neurons have been reported for amphibians, reptiles and birds (e.g. Hubl, Mohneke and Schneider, 1977; Walkowiak, 1980; Smolders and Klinke, 1984; Schermuly and Klinke, 1985). In guinea pigs, a correlation between the temperature and the characteristic frequency of neurons was not observed (Gummer and Klinke, 1983), but a loss in sharpness and an increase in threshold did occur. In the non-DS compensating bat *Myotis lucifugus*, temperature reduction led to a reversible decrease of sensitivity of the N1 response to all frequencies, with a greater effect observed at higher frequencies (Harrison, 1965).

In humans, a variation of the characteristic frequency of spontaneous otoacoustic emissions observed during menstrual or diurnal cycles, as well as during fever, has been discussed (Wit, 1985) and supported (Wilson, 1986; O'Brien, 1994) as an effect of body temperature. Bell (1982), however, found no body temperature effect, and suggested that a more likely candidates were changes in hormonal or cardiovascular activity. Wynn (1972) found small variation in estimation of absolute pitch in both women and men, due to hormonal cycle or illness. Strong evidence that hormones play a role in ear activity is the presence of beta1-adrenergic receptors in the Organ of Corti, which Fauser et al. (2004) found in gerbils. They concluded that the sympatric innervation may enhance the potassium (K^+) efflux in the inner and outer hair cells. Therefore, we cannot exclude that in DS-compensating bats, additional physiological factors may have an influence on the resonance properties of the cochlea.

Conclusions

The observed increase of f_{rest} in warm-up phases of *H. armiger* reflects a change in the temperature-dependent controlled process variable of the audio-vocal control system, i.e. the cochleo-topic activation state of the foveal resonance area in the cochlea. A concomitant change in emission frequency occurs if the state that is reported to higher foveal centres deviates from the central set-point condition, owing to increases in body, and therefore cochlea, temperatures. We propose to generalize our conclusions to all other DS-compensating hipposiderids and rhinolophids bats, and also to the DS-compensating mormoopid bat *P. parnellii*, based on findings that this species reacts in a comparable way to changes in body temperature. The cochleo-topic organization of the feedback control system guarantees an undisturbed function of DS compensation independent of physiologically determined changes of emission frequency.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: D.S., A.D., H.-U.S.; Methodology: D.S., A.D., H.-U.S.; Formal analysis: D.S.; Investigation: D.S.; Resources: A.D., H.-U.S.; Writing - original draft: D.S., H.-U.S.; Writing - review & editing: D.S., A.D., H.-U.S.; Visualization: D.S.; Supervision: A.D., H.-U.S.; Project administration: A.D., H.-U.S.

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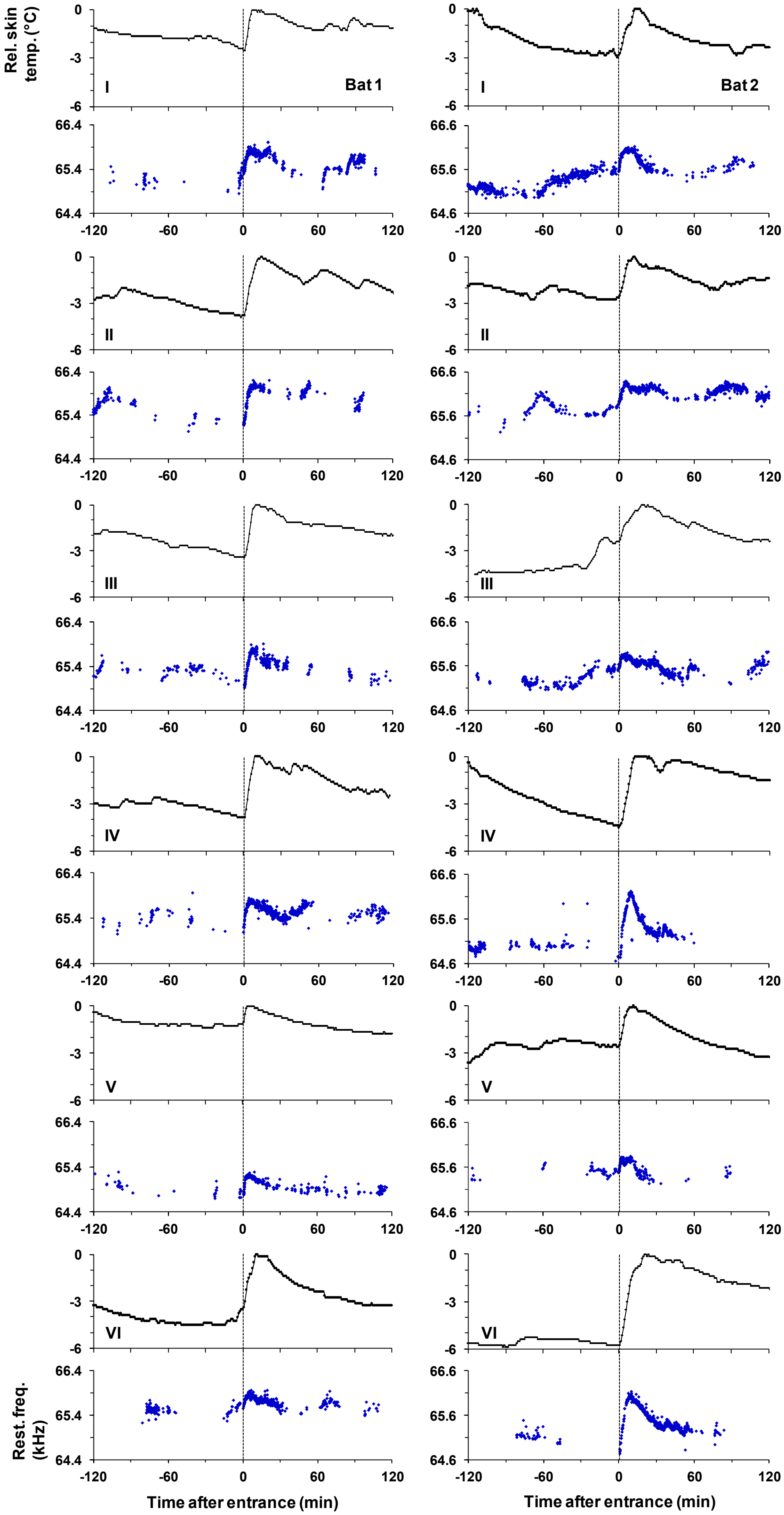
Rel. skin temp. (°C)
Rest. freq. (kHz)
Bat 1
Bat 2
I
II
III
IV
V
VI
0
-3
-6
66.4
65.4
64.4
66.6
65.6
64.6
-120
-60
0
60
120
Time after entrance (min)

Journal of Experimental Biology: doi:10.1242/jeb.243569

Supplementary information

Fig. S1. Relative skin temperature (Rel. skin temp.) and resting frequency (Rest. freq.) over a time course of two hours before and after activation by the experimenter. For each bat, six activations are shown. Skin temperature is depicted relative to the maximum temperature reached after each activation. The vertical lines mark the instant of time when the experimenter entered the husbandry room. Each frequency value represents the resting frequency in a five-second slot

Author information and contribution from others to chapter 2

The first experiment “*Measurement of Doppler shift compensation*” of chapter 2 was planned by me together with my advisers Annette Denziner and Hans-Ulrich Schnitzler, and I conducted the experiments, recorded, analysed and edited the data. The second experiment of chapter 2 “*Measurement of the behavioural audiogram*” was also planned by Annette Denzinger and Hans-Ulrich Schnitzler and performed by Katrin Kost (geb. Schmuck) who measured and analysed the data and presented them in her diploma thesis “Die Pinnareflexschwelle bei *Rhinolophus paradoxolophus*“. I wrote the first draft of the manuscript, which is presented in chapter 2 “Doppler shift compensation and audiogram of the low frequency horseshoe bat *Rhinolophus paradoxolophus* are comparable to that of other rhinolophids”. The current version of the manuscript was written by me, Annette Denzinger and Hans-Ulrich Schnitzler and will be submitted soon with all them and Katrin Kost as co-authors. Animals and technical resources were provided by Hans-Ulrich Schnitzler and Annette Denzinger.

Chapter 2

Doppler shift compensation and audiogram in the low frequency horseshoe bat *Rhinolophus paradoxolophus* are comparabe to that of other rhinolophids

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Doppler shift compensation and audiogram of the low frequency horseshoe bat *Rhinolophus paradoxolophus* are comparable to that of other rhinolophids

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Abstract

Flutter detecting foragers require specific adaptations of the transmitter and the receiver to process flutter information in the echoes from insects. These adaptations have been shown for only a few high duty cycle bats though it was concluded that all flutter detecting foragers have been adapted in a similar way for processing of flutter information. A common trait of the transmitters should be Doppler shift compensation (DSC), which keeps the echoes from targets ahead constant at a reference frequency (f_{ref}), so that the carrier frequency for insect echoes with flutter information is near f_{ref}. A common trait of the receivers should be an auditory fovea around $f_{ref,}$ resulting in foveal areas in the hearing system with many sharply tuned neurons with best frequencies near f_{ref}. Here we show for *Rhinolophus paradoxolophus*, an allometric outlier that uses a low frequency echolocation system with signal frequencies of about one octave below the frequency of other similar-sized rhinolophids, that transmitter and receiver are organized in a similar way as postulated for all flutter detecting foragers. The performance of DSC, measured as the ability to keep the echo frequency constant at f_{ref}, had a similar quality as that of other flutter detecting foragers. The foveal organization of the hearing system was indicated by a minimum of the behavioural auditory threshold, which was tuned to f_{ref} as estimated from the coupled f_{rest} of the tested individual. Further, we showed for the first time in a rhinolophid a parallel variation of the coupled f_{rest} and f_{ref}. Our results suggest that the outlier *R. paradoxolophus* with its low frequency echolocation system is well equipped for fluttering target detection by a tight match of its transmitter and receiver properties.

Introduction

Echolocation and powered flight allow bats to exploit a high diversity of diets and foraging habitats at night. Their echolocation and motor systems are adapted to where they forage and how they find and acquire prey. The strongest constraints on the echolocation and motor systems are set by the distances between bat, prey, and background (Schnitzler and Kalko, 1998; Schnitzler and Kalko, 2001; Schnitzler et al., 2003; Denzinger and Schnitzler, 2013). Bats that forage for prey in narrow space have to recognize prey echoes among many clutter echoes, which is basically a pattern recognition task (Denzinger et al., 2018). Narrow space foragers have evolved three different echolocation strategies to find prey. Narrow space passive gleaning foragers use prey generated cues to find prey, narrow space active gleaning foragers evaluate spectral–temporal patterns in prey–clutter echo complexes and narrow space flutter detecting foragers assess flutter information in the echoes from fluttering prey, which consists of amplitude and frequency modulations caused by the wing movements of prey insects. All members of the families of Rhinolophidae and Hipposideridae, and the phylogentically more distant mormoopid *Pteronotus parnellii* belong to the narrow space flutter detecting foragers (Denzinger and Schnitzler, 2013).

During evolution, transmitter and receiver of flutter detecting foragers have been adapted to evaluate flutter information in echoes from fluttering prey (for reviews, see Schnitzler and Ostwald, 1983; Schnitzler and Denzinger, 2011). A characteristic property of the transmitter in flutter detecting foragers is the use of multi-harmonic echolocation signals emitted at a high duty cycle, which consist of a long constant-frequency (CF) component followed by a downward frequency-modulated (FM) terminal part. The second harmonic contains the main energy (CF_2). Due to the high duty cycle flutter detecting foragers have also been classified as high duty cycle bats (Fenton et al., 2012). Another characteristic transmitter property is the Doppler shift compensation (DSC) (Rhinolophidae: Schnitzler, 1968; *P. parnellii*: Schnitzler, 1970; Hipposideridae: Gustafson and Schnitzler, 1979). With an audio-vocal control feedback system, these bats compensate for Doppler shifts (DS) induced by their own flight movement, by keeping the CF_2 of echoes from targets ahead (f_{echo}) constant at a reference frequency (f_{ref}), which is defined as the averaged f_{echo} (Schnitzler, 1973). Small deviations from f_{ref} of not more than 0.1-0.2% have been measured (Schnitzler and

Denzinger, 2011). The value under control (f_{echo}) is maintained at the reference value (f_{ref}) over the whole range of the bats flight speed, indicating a high quality of the DSC system (Schnitzler, 1973; Schnitzler and Denzinger, 2011; Schoeppler et al., 2018). The f_{ref} is about 150-200 Hz above the CF_2 of stationary bats, the so-called resting frequency (f_{rest}) (reviewed in Schnitzler and Denzinger, 2011). This difference is termed offset (Schuller et al., 1974). Although f_{ref} and f_{rest} could change over time, e.g., owing to different body temperatures, it is a common feature of the transmitter system that f_{rest} and f_{ref} are coupled (Huffman and Henson, 1993; Schoeppler et al., 2018).

If the long CF call components hit a fluttering insect, the returning echoes contain short and strong amplitude and frequency modulation peaks, so called glints that arise when the reflecting wing is perpendicular to the impinging sound waves (Schnitzler, 1978; Schnitzler et al., 1983). In many insect species the glints are produced in the rhythm of the wing beat (Kober and Schnitzler, 1990). This flutter information enables bats to distinguish prey echoes from unmodulated background echoes (Schnitzler and Henson, 1980). The information contained in the glint pattern even allows selective foraging (Goldman and Henson, 1977; Trappe and Schnitzler, 1982; Schnitzler and Flieger, 1983; von der Emde and Schnitzler, 1986; Link et al., 1986; von der Emde and Menne, 1989; von der Emde and Schnitzler, 1990; Koselj et al., 2011).

The receiver, which corresponds to the the auditory system of flutter detecting foragers, is especially adapted to process flutter information in echoes from flying insects. The auditory fovea, an expanded frequency representation around f_{ref} on the basilar membrane (*R. ferrumequinum*: Bruns, 1976; Bruns and Schmieszek, 1980; *R. rouxii*: Vater et al., 1985; *P. parnellii*: Kössl and Vater, 1985; *Hipposideros lankadiva*: citated in Neuweiler, 1990), leads to afferent projections in higher foveal areas with an overrepresentation of sharply tuned neurons near f_{ref} (*R. ferrumequinum*: Schuller and Pollak, 1979; *Hipposideros speoris*: Rübsamen et al., 1988; *P. parnellii*: Pollak and Bodenhammer, 1981). These neurons have the function to decode the flutter information in insect echoes and to deliver relevant feedback information to the audio-vocal control system, which determines the frequency of the emitted signals (for reviews, see Neuweiler et al., 1980; Schnitzler and Ostwald, 1983; Metzner, 1989; Schnitzler and Denzinger, 2011).

The foveal organization of the auditory system of flutter detecting foragers is also reflected in the behavioural and neurophysiological audiograms of these bats, which exhibit a distinct threshold minimum at the f_{ref}, a lower well-defined maximum at the lowest emission frequencies of DSC bats, and a rise of the threshold above f_{ref} with a sustained high threshold at higher frequencies (rhinolophids: Neuweiler, 1970; Schnitzler et al., 1971; Long and Schnitzler, 1975; Schnitzler et al., 1976; Schuller, 1980; Henson et al., 1985; Taniguchi, 1985; *P. parnellii*: Grinell, 1970; Pollak et al., 1972; Suga et al., 1975; Henson et al., 1985; hipposiderids: Grinnell and Hagiwara, 1972; Schuller, 1980; Neuweiler et al., 1984). In the frequency range of the FM and of the social calls, the audiograms additionally show broadly tuned minima (Long and Schnitzler, 1975).

The tight match between transmitter properties such as DSC, which adjusts the carrier frequency of insect echoes with flutter information at f_{ref}, and receiver properties such as the tuning of the auditory fovea and the resulting minimum in the audiograms at f_{ref} is an indispensable prerequisite for the evaluation of flutter information. This match, however, is postulated on the basis of studies on a very few species of flutter detecting foragers. The direct match of transmitter and receiver properties is only proved for the rhinolophids *R. ferrumequinum* (Long and Schnitzler, 1975; Schnitzler et al., 1976), *R. ferrumequinum nippon* (Taniguchi, 1985); *R. rouxii* (Henson et al., 1985) and the mormoopid *P. parnellii* (Henson et al., 1980; Henson et al., 1985). Hence, for most species some details on transmitter or receiver properties have been published, but there is a lack of prove that transmitter and receiver properties are matched. This accounts particularly for hipposiderids. In *Asellia tridens* for example, only data from a precise DSC is available (Gustafson and Schnitzler, 1979). In *Hipposideros speoris* and *H. bicolor*, the results from DSC and audiograms do not clearly coincide (Schuller, 1980). Similarly, in less excessively studied rhinolophids, some details have been published without assessment of the whole transmitter-receiver-system. For example, in *Rhinolophus philippinensis* foveal neurons are tuned to 30-33 kHz, with $Q_{10\,dB}$ values of up to 65 (Jen and Suthers, 1982).

Nevertheless, we hypothesize for all other species of flutter detection foragers that transmitter and receiver properties are matched. To test this hypothesis, we chose the horseshoe bat species *Rhinolophus paradoxolophus*, which differs in many characters from most rhinolophids. It belongs to the south-east Asian *philippinensis* group, which forms a subgroup within the rhinolophids that emits echolocation signals with CF_2 distinctly lower than predicted by allometry (Francis and Habersetzer, 1998; Zhang et al., 2018). *R. paradoxolophus* is a fairly small species with a body mass around 9.5 g, a mean forearm length of 51 mm and a very low f_{rest} of around 28.5 kHz, which is about an octave lower than that of other horseshoe bats of the same size. We assume that *R. paradoxolophus* uses their low frequency echolocation signals in a similar way as other flutter detecting foragers to aquire fluttering prey in narrow space. Further, we expect that the transmitter and receiver properties of *R. paradoxolophus* match in a similar way as in other rhinolophids. To determine the transmitter properties we trained the bats to perform a landing task and measured the precision of DSC and the coupling of f_{rest} to f_{ref}. The receiver properties were determined by measuring the behavioural audiogram of *R. paradoxolophus*. Therefore, we identified the lowest amplitude over the frequency range of 15 to 100 kHz, which just elicited a pinna reflex, also known as Preyer reflex. The behavioural auditory threshold of flutter detecting foragers is a good indicator for the foveal organization of the auditory receiver (Schnitzler and Denzinger, 2011). A distinct minimum at f_{ref} suggests the presence of an auditory fovea and higher foveal centres with many sharply tuned neurons with best frequencies near f_{ref} specialized to decode flutter information in insect echoes. We hypothesized that – independent of the low frequency range – the course of the auditory threshold curve should be similar to that of other rhinolophids. Finally, we hypothesized that f_{ref} as determined by DSC should predict a minimum in the behavioural audiogram at f_{ref}, thus confirming the close match between transmitter and receiver adaptations also for the low frequency outlier *R. paradoxolophus*.

Materials and methods

Ethical statement

All experiments were performed in accordance with the German Animal Welfare Legislation. The license to keep *Rhinolophus paradoxolophus* was issued by the responsible agency (Regierungspräsidium Tübingen, Germany).

Animals and husbandry

The three adult, male *Rhinolophus paradoxolophus* (Bourret 1951) were captured in Northern Vietnam with permission No. 192/STTNSV from May 13th, 2009 granted to the Vietnamese Institute of Ecology and Biological Resources in Hanoi. At the University of Tübingen (Germany) bats were housed in a tent (2.3×1.4×1.25 m) under a constant temperature of 26±2 °C, a constant humidity of 60±5%, and a light:dark cycle of 12:12 h (dark from 8 am to 8 pm). The bats were fed with mealworms (*Tenebrio molitor* larvae), which were kept in Korvimin® (WDT eG, Germany) one day before fed to the bats to guarantee a balanced calcium-phosphate-ratio. Two times per month, the food was additionally supplemented with vitamins (Nutri-Cal® Albrecht GmbH, Germany) and fatty acids (Efaderm® aristavet GmbH & Co., Germany). Water was offered *ad libitum*. All experiments were conducted at the beginning of the dark phase of the daily cycle.

Experiment 1: Measurement of Doppler shift compensation

Training procedure and data acquisition

Experiments were conducted in a flight room (6×3.5×3 m). Bats were rewarded with mealworms for correct behaviour. Training sessions lasted between 30 and 45 min, and ended when the bat was satiated, usually after ~10 mealworms. After the training sessions, the bats had *ad libitum* access to food in the housing tent for the rest of the dark phase to make sure, that every bat could fully supply itself with food. At days without training, the bats had *ad libitum* access to food. The weight of the bats was daily monitored.

Bats were trained to fly from a starting bar, positioned 1.5 m above the floor, to a landing grid attached to the ceiling at a height of 3 m. The direct distance between start and landing was 3.7 m (Fig. S1). The echolocation signals were picked up with a custom-made ultrasonic microphone (University of Tübingen, Germany; flat frequency response: 15–75 kHz ±2 dB SPL), which was positioned behind the landing grid and stored as wav files at a sampling rate of 480 kHz, a resolution of 16 bits, and an amplification of +20 dB (PC-Tape system, University of Tübingen, Germany). Sound recordings were synchronized with two infrared cameras (Sanyo IR CCD, Panasonic, Osaka, Japan) that recorded the flight behaviour at a rate of 50 half-frames/s. Each half frame was illuminated for 1 ms with two infrared strobe flashes (University of Tübingen, Germany) positioned on the cameras. The video data were stored on tapes using camcorders (Sony, DCR-TR V50E, Tokio, Japan). After digitization, we reconstructed the three-dimensional flight path (SIMI® Motion Reality Motion Systems, 7.5.293, Germany) and calculated the flight speed of the bat (reconstruction error of 1.0 cm on average and 2.8 cm at highest).

Data analysis

Sound analysis was conducted with the custom-written software Selena (Animal Physiology, University of Tübingen, Germany). For precise frequency measurements, which were necessary to determine DSC, signals were analysed with an FFT of 8192 points, a dynamic range of 90 dB, Blackman window and zero padding and displayed in the frequency range of 25–31 kHz as sonagrams in a 200 ms long window with 512×512 pixels resulting in a frequency resolution of 11.5 Hz. CF_2 was measured at peak amplitude of the CF component. Other signal parameters were measured with an FFT of 512 points and displayed as sonograms in the frequency range of 0–50 kHz in a 120 ms long window with 512 x 512 pixels resulting in a frequency resolution of 97.9 Hz and a time resolution of 0.23 ms. The beginning and end of the signal was defined at 30 dB below best amplitude and the end of the initial frequency modulated component (iFM) and the beginning of the terminal frequency modulated component (tFM) was determined 1% (285 Hz) below the CF_2 with a custom Matlab® routine (MathWorks® USA, 2013b), written by Peter Stilz (University of Tübingen). Pulse interval was defined as the time from the beginning of one signal to the beginning of the signal before and duty cycle as the percentage of time filled with signal.

We determined the root mean square sound pressure level (rms SPL) of the iFM, CF and tFM relative to full-scale of the signals recorded at the microphone with a custom Matlab® routine (INAT, written by Peter Stilz, MathWorks® USA, 2014b), up to the first signal of the terminal group. We excluded the terminal group, because not all signals in the terminal group were oriented towards the microphone due to the landing turn.

The resting frequency (f_{rest}) was measured prior to each flight, as averaged CF_2 of the last 20 calls before take-off. The CF_2 of the emitted signals (f_s) was calculated from the microphone frequency (f_m = frequency recorded at the microphone) by evaluating the

DS produced by the approaching bat according to its flight speed (v_b) and the velocity of sound (c = 343 m/s) using the equation (1) published by Schnitzler (1973):

$$\mathrm{fs} = \mathrm{fm} \times \frac{(\mathrm{c}-\mathrm{vb})}{\mathrm{c}} \tag{1}$$

In situations where bats did not fly straight towards the microphone (Fig. S1), the DS was lower than predicted from the bat's flight speed. Therefore, we measured the angle α between flight and microphone direction and used the cosine of α to correct the DS and to calculate the emission frequency with:

$$\mathrm{femission} = \mathrm{fs} - \mathrm{DS} \times \cos(\alpha) \tag{2}$$

The echo frequency (f_{echo}) from stationary targets was calculated with equation (3) by adding two DS (Schnitzler 1973):

$$\mathrm{fecho} = \mathrm{femitted} \times \frac{(\mathrm{c}+\mathrm{vb})}{(\mathrm{c}-\mathrm{vb})} \tag{3}$$

The reference frequency (f_{ref}) was determined as the averaged echo frequency of each flight, and the difference between f_{ref} and f_{rest} described the offset.

Statistical analysis

Statistical analysis was performed in JMP® (SAS Institute GmbH, Heidelberg, Germany). For each bat we analysed 10 flights. Each flight was classified into orientation and approach flight. The start of the approach was defined, when the bat started to emit groups of calls until landing. We checked for normal distribution using normal quantile plots. In case of normal distribution, we compared the beginning of the approach between bats, using ANOVA and Tukey-Kramer test. Nonparametric signal parameters were tested with Kruskal-Wallis tests for differences between the bats. The iFM was only present in the first signal of a group except for bat 2. Therefore, we only used the iFM of the first signal of a group in all bats for statistical analysis. Further, we tested the coupling of f_{rest} and f_{ref} in each bat with a Pearson correlation. We measured the precision of DSC by calculating the deviation of the f_{echo} of each call from the fref of the corresponding flight. We displayed this deviation in relation to the flight speed. We calculated means of deviations within flight speed classes of 0.25 m/s for each bat, i.e.

15, 13, and 14 classes for bats 1, 2, and 3, respectively (Fig. 6). These means of the three bats between flight speed classes were compared with an ANOVA and Tukey-Kramer test.

Experiment 2: Measurement of the behavioural audiogram

Training procedure and data acquisition

The behavioural audiogram was measured by determining the threshold SPL, which just elicited a Preyer reflex at different frequencies. The Preyer reflex is a characteristic ear twitch in response to a weak auditory stimulus and can only be measured in a calm and relaxed animal. The study was carried out with one bat (bat 1). Measurements were conducted in a recording box (60×45×36 cm) in the flight room. The bats were familiar with the box as it was used to train them to feed on mealworms when they came to the animal facility and to keep bats during cleaning their enclosure. For the measurements, the box was covered with foam and a soft grid (12.5×12.5 cm) was attached to the ceiling to offer a preferred resting site. In the box bats had free access to water. Food was offered regularly. The video system, the speaker and the microphone were positioned at one side of the box in alignment to the bat's position on the resting grid.

Prior to transferring the bat into the recording box, it was allowed to fly for 1 to 2 h together with conspecifics in the flight room. In the recording box, the bat calmed down within a few minutes. Stimulus presentation started when the bat rested calmly at the grid without moving its ears and without emitting echolocation signals. Sessions ended, when the bat started to flap its wings and move around.

Stimulus generation and video recordings

For stimulus generation we used a custom Matlab® routine (MathWorks® USA, R2011b) written by Manfred Kössl (University of Frankfurt). The pure tone stimulus was generated with a sound card (Quartet® Infrasonic, multi-purpose 4 x 4 channel 24 bit/192 kHz, Serial No. SMC080501737; Newegg, CA, USA), amplified (Krohn-Hite®, Model 7500, MA, USA) and played back through a custom-made loudspeaker (University of Tübingen, Germany). The loudspeaker was calibrated (Audio Wave Analyzer, Rhode &

Schwarz, 1/8 inch Brüel & Kjær® microphone, Nærum, Denmark) and had a flat frequency response from 15–110±1.5 kHz up to 95 dB rms SPL re 40 cm in front of the speaker, which corresponded to the distance between loudspeaker and bat in the setup.

Stimuli always had a duration of 30 ms with a slope of 1.5 ms. Thresholds were measured at frequencies from 15–95 kHz in 5 kHz steps except around the 2nd and 4th harmonic of the echolocation signals (CF_2 and CF_4), where smaller steps were used (measured frequencies: 24.3 kHz, 26.3 kHz, 28.3 kHz, 28.55 kHz, 28.8 kHz, 29.05 kHz, 29.3 kHz, 29.8 kHz, 30.3 kHz, 31.3 kHz, 32.3 kHz, 36 kHz, 58.1 kHz, 58.6 kHz, 59.1 kHz, 62.6 kHz, 63.6 kHz, 64.6 kHz and 67.4 kHz). In total, the threshold was determined for 35 frequencies. Except around CF_2, each frequency threshold was measured with an ascending staircase procedure at least three times, starting with stimulus amplitude of 30 dB SPL. The low amplitude ensured that no startle response occurred, even at frequencies with lower thresholds. Amplitude was increased in 5 dB steps until the pinna reflex was elicited. If the bat already responded at 30 dB, we reduced the amplitude to sub-threshold level. Around the CF_2 we started with stimulus amplitude of 5 dB SPL and used 1 dB steps. Around the CF_4 we also measured in 1 dB steps. To avoid habituation, we used the highest possible difference for consecutive stimulus frequencies, and stimulus intervals of at least 2 min.

Data analysis

At the beginning of each session resting signals were recorded for 5 to 10 s. Resting signals, stimulus and the behaviour of the bat were recorded with the PC-tape system and the synchronized infrared video system as described above but with only one camera equipped with a zoom objective (Computar TV Zoom Lens, H16Z7516M, 7.5-120 mm; NC, USA). Video tapes were digitized and analysed in SIMI° Motion (SIMI® Motion Reality Motion Systems, 7.5.293, Germany). For analysis of the pinna reflex the distance between a fixed point on the head between the two ears and the tip of the respective ear was measured at each half frame (Δt=0.02 ms and Δd=1.2 mm). A positive reaction of the bat was defined as a pinna movement of at least 0.01 in the coordinates of the SIMI program which corresponded to 1.2 mm, calibrated with ear length (Fig. S2). Pinna movement was analysed from 0.2 s before to 1 s after the stimulus. Each frequency of the audiogram was measured up to five times and displayed as a mean with standard error (±SEM).

Results

Experiment 1

Echolocation behaviour in flight

All *R. paradoxolophus* flew on a stereotyped path from the starting bar to the landing grid, bat 1 and bat 2 in an arc slightly to the right and bat 3 directly or in an arc to the left (Fig. S1). During orientation flight, before reacting to the landing site, bats emitted long signals with the main energy in the second harmonic (Fig. 1A). Signals started with a short iFM component, followed by a long CF and a short tFM component (Fig. 1B). The calls had an average duration of 45–50 ms, an average pulse interval of 86–87 ms and an average duty cycle of 49–53% (Table 1, Fig. 2A, C). The tFM had an average duration of 2–3 ms and a bandwidth of 1.9–2.9 kHz. In all bats, the duration of the iFM from 2–2.5 ms was shorter and the bandwidth with values from 1.2–2.1 kHz was lower than that of the tFM (e.g., in bat 1: Fig. 2B, D). All signal parameters during orientation flight differ significantly between the bats (Kruskal-Wallis $\chi^2_{(2)} \geq 9.7$; $p \leq 0.0079$).

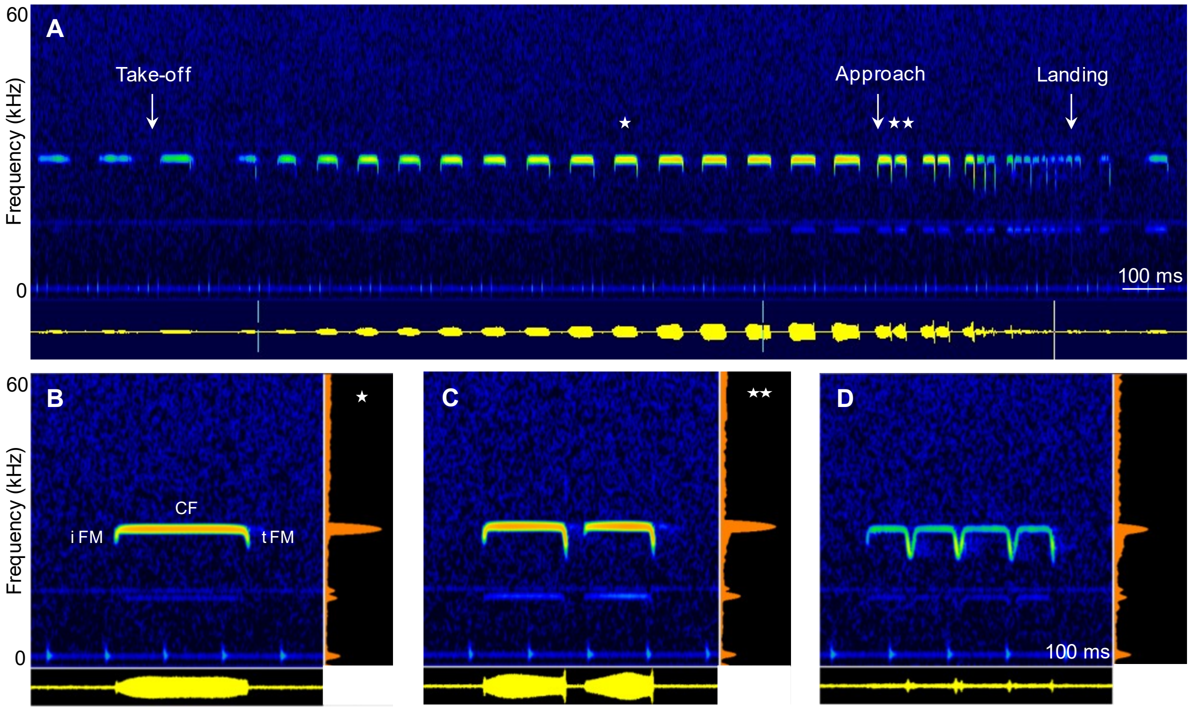

Fig. 1: Sonagram and oscillogram of an echolocation sequence of bat 1 flying to a landing grid (A) and of representative signals with averaged power spectra (B-D). Signals in (B) and (C) are taken from A (marked with asterisks). (D) shows a group of four signals of bat 2 with untypical long iFM components emitted mostly within groups of more than two signals. iFM = initial frequency-modulated component, CF = constant-frequency component and tFM = terminal frequency-modulated component.

During approach, signals were arranged in groups. The first group always contained two signals and signals per group increased up to four signals in the further course of the approach. The approach ended with a long terminal group of seven to ten signals on average and 13 signals maximally. The approach behaviour started on average 620 ms or 1.11 m before landing. With 432 ms bat 1 started later than in the other two bats (687 ms and 740 ms) [F(2,27) = 12.2; p = 0.0002, Tukey-Kramer p<0.05]. It emitted just four groups of signals, while the two others produced seven groups of signals on average. Nevertheless, the distance at which the approach began was similar, with 0.9 m before landing in bat 1 and 1.2 m in bat 2 and 3 [F(2,27) = 3.3, p = 0.053].

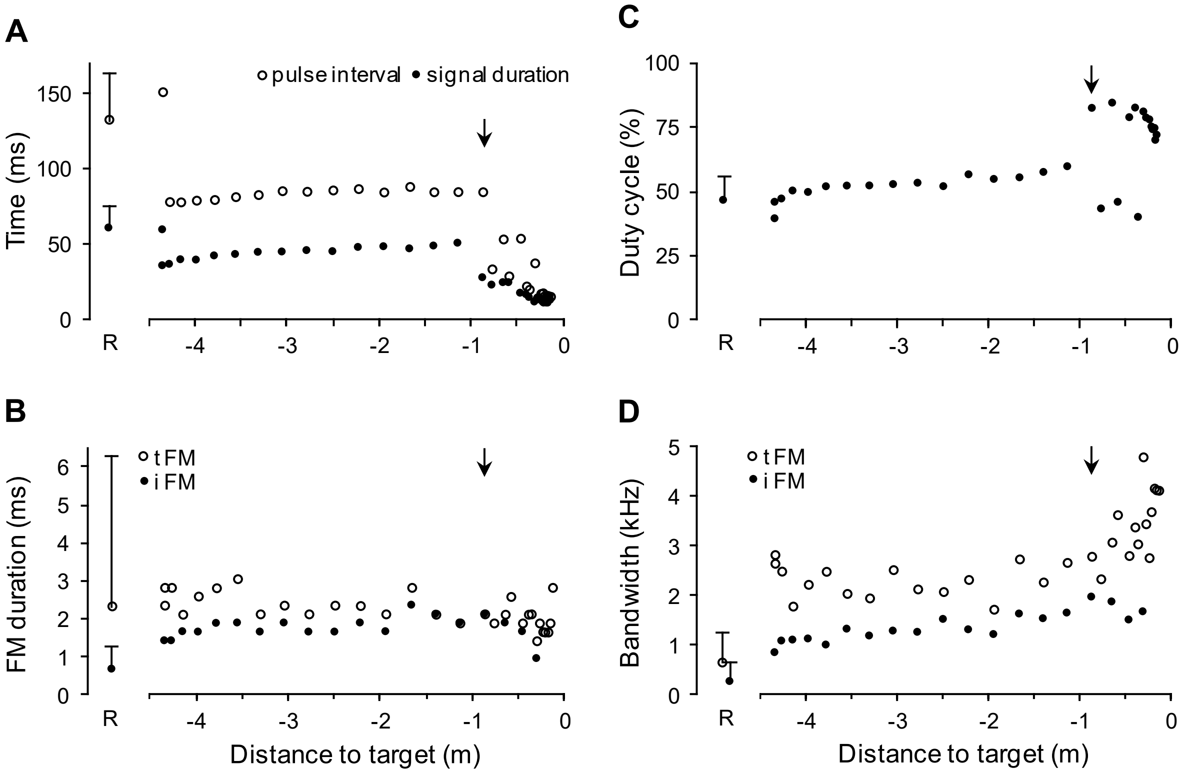

Fig. 2: Signal parameters of calls from the echolocation sequence shown in Fig. 1A. R indicates the mean+s.d. of the resting signals (n=20) emitted prior to take off. The approach behaviour started at 87 cm before landing (marked with an arrow) and ends with the terminal group, starting 31 cm before landing.

With the beginning of the approach, signal duration and pulse interval decreased and duty cycle increased. In the terminal group, a signal duration of 12 ms, a pulse interval of 17-20 ms, and a duty cycle of 72–84% was measured; the tFM was shorter in all bats, had a higher bandwidth (e.g., in bat 1: Fig. 2B, D), and was thus more steeply modulated than during orientation flight. On average, tFM bandwidth increased between 0.6 kHz (bat 3) and 2.9 kHz (bat 2). In contrast to the tFM, the iFM was only present in the first signal of a group (Fig. 1C) except for bat 2, who produced a distinct iFM in subsequent signals mainly within groups of three or more calls (Fig. 1D). In the terminal group, the duration of the iFM was shortened by up to 0.8 ms and the bandwidth of the iFM was higher in bat 1, but in two bats (2 and 3) lower than in the orientation flight. The signal duration and the duration of the iFM of the terminal group were similar in all bats (Krsukal-Wallis $\chi^2_{(2)} \leq 5.8$; $p \geq 0.0562$), but pulse interval, duty cycle, duration of tFM and bandwidth differed between individuals (Kruskal-Wallis $\chi^2_{(2)} \geq 10.8$; $p \leq 0.0046$)

(Table 1).

Table 1: **Echolocation signal parameters during orientation and approach flight** (mean±s.d. (N) and p-values (Kruskal-Wallis)).

	Bat	Orientation flight	Approach flight	
			before terminal group	terminal group
Signal duration (ms)	1	46.3 ± 6.4 (188)	21.8 ± 4.7 (77)	12.3 ± 1.5 (94)
	2	45.0 ± 8.5 (168)	23.4 ± 5.9 (149)	12.0 ± 2.0 (103)
	3	50.2 ± 11.4 (145)	25.1 ± 7.1 (153)	12.4 ± 1.9 (68)
		$p < 0.0001$	$p = 0.0018$	$p = 0.1626$
Pulse interval (ms)	1	86.4 ± 9.5 (178)	42.7 ± 21.6 (77)	18.9 ± 8.5 (94)
	2	85.9 ± 13.4 (158)	40.1 ± 20.6 (149)	16.5 ± 7.1 (103)
	3	87.2 ± 20.2 (135)	45.0 ± 21.8 (153)	20.1 ± 7.7 (67)
		$p = 0.0079$	$p = 0.0527$	$p < 0.0001$
Duty cycle (%)	1	50.4 ± 13.6 (188)	58.6 ± 33.9 (77)	76.0 ± 5.4 (84)
	2	49.1 ± 15.0 (168)	68.9 ± 28.4 (149)	84.4 ± 10.6 (93)
	3	52.7 ± 18.9 (145)	64.6 ± 26.8 (153)	71.7 ± 7.4 (58)
		$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
Terminal FM duration (ms)	1	2.3 ± 0.4 (183)	2.0 ± 0.2 (75)	2.0 ± 0.7 (51)
	2	2.6 ± 0.5 (164)	2.3 ± 0.3 (146)	2.2 ± 0.3 (45)
	3	3.1 ± 0.9 (142)	2.0 ± 0.2 (149)	1.8 ± 0.3 (28)
		$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
Terminal FM bandwidth (kHz)	1	2.07 ± 0.48 (183)	2.97 ± 0.46 (125)	3.43 ± 0.53 (51)
	2	1.91 ± 0.58 (164)	3.58 ± 0.94 (191)	4.79 ± 0.78 (45)
	3	2.94 ± 0.63 (142)	3.56 ± 0.41 (177)	3.53 ± 0.79 (28)
		$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
Initial FM duration (ms)	1	1.7 ± 0.4 (178)	1.9 ± 0.2 (33)	1.6 ± 0.7 (8)
	2	2.1 ± 0.4 (153)	2.1 ± 0.3 (61)	1.3 ± 0.3 (8)
	3	2.5 ± 0.6 (135)	2.4 ± 0.3 (62)	2.1 ± 0.7 (4)
		$p < 0.0001$	$p < 0.0001$	$p = 0.0562$
Initial FM bandwidth (kHz)	1	1.22 ± 0.36 (178)	1.78 ± 0.24 (33)	1.47 ± 0.40 (8)
	2	1.66 ± 0.43 (153)	1.99 ± 0.28 (61)	0.99 ± 0.48 (8)
	3	2.07 ± 0.55 (135)	2.72 ± 0.22 (62)	1.95 ± 0.29 (4)
		$p < 0.0001$	$p < 0.0001$	$p = 0.0046$

The amplitude ratio between the CF component and the tFM component measured at the microphone differed between orientation flight and approach. In the beginning of the orientation flight, the maximum amplitude was always in the CF. However, at a distance of 1.25-1.5 m before landing, the relative rms SPL of the tFM increased and reached the same level as the relative rms SPL of the CF. In the approach, the relative rms SPL of the tFM was always higher than the that of the CF (Fig. 1B, C and 3A). At the microphone, the relative rms SPL of the tFM was almost constant indicating, that bats reduced the emission SPL of the tFM with 6-7 dB per halving distance (dB/hd) during approach. In contrast, the relative rms SPL of the CF measured at the microphone decreased during approach. Bat 2 and bat 3 reduced the emission SPL of the CF by 9 dB/hd and bat 1 by 10 dB/hd. The relative rms SPL of the iFM in bat 1 and bat 2 was always below the CF and tFM, slopes of -1.2 and -0.5 in approach phase indicating a reduction of emission SPL by ca. 7 dB/hd. In bat 3, the relative rms SPL of the iFM exceeded the CF in approach phase and ran almost parallel to the CF, which indicated a slightly higher reduction of the emissions SPL compared to the other bats by approx. 8 dB/hd (Fig. 3B).

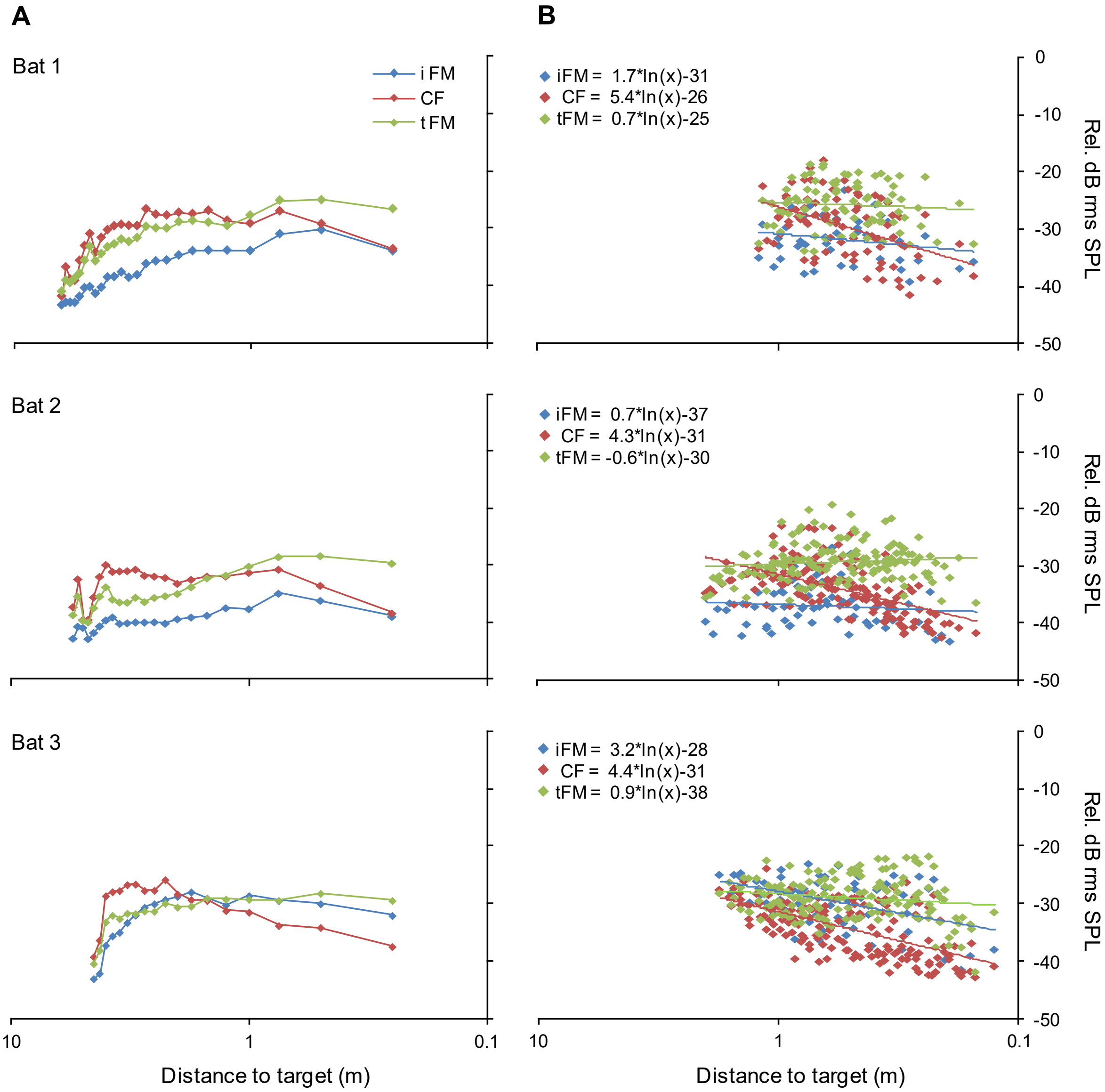

Fig. 3: Relative root means square sound pressure level (rms SPL) of the iFM, CF and tFM component referred to full scale of the recording system measured at the microphone. (A) averaged relative dB rms SPL of all flights (n=10) per bat in 0.25 m distance classes. (B) relative dB

rms SPL of all approach signals with regression lines and functions.

Doppler shift compensation system and frequency variation

R. paradoxolophus emitted echolocation calls with a rather low species-specific CF$_2$, which is typical for members of the *philippinensis* group. The bats had individual mean CF$_2$ of 28.5 kHz, 28.35 kHz and 28.44 kHz in resting signals, termed f_{rest}, measured over the whole recording time of three weeks. In this time period the f_{rest} varied by up to 60-90 Hz between days, but before each flight the averaged standard deviations were very small with 20 Hz (bat 2) or 30 Hz on average (bat 1 and bat 3) and 40 Hz at maximum.

In flight to a stationary target, *R. paradoxolophus* lowered the emission frequency according to their own flight speed, to maintain the echo frequency (f_{echo}) constant at a reference frequency (f_{ref}) (Fig. 4). The averaged standard deviation of f_{echo} was 60 Hz (bat 1: 70 Hz, bat 2: 60 Hz, bat 3: 50 Hz), and ranged from 40–120 Hz. The offset between the f_{rest} and the f_{ref} was 70 Hz (bat 1: 80 Hz, bat 2: 70 Hz, bat 3: 60 Hz). A variation of f_{ref} between days of 70-80 Hz at maximum in bat 1 and 2 and 30 Hz in bat 3 was observed.

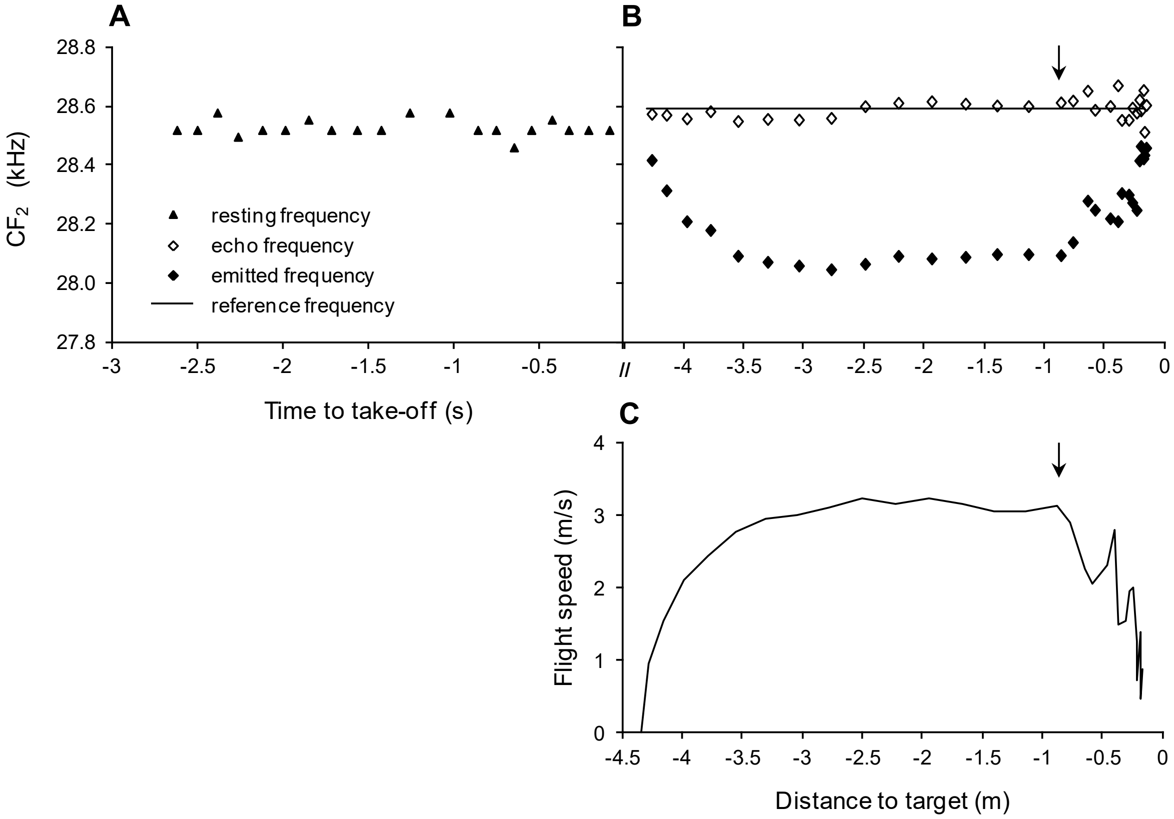

Fig. 4: Doppler shift compensation of the representative flight shown in Fig. 1A. (A) CF_2 of the last 20 echolocation signals before take-off (f_{rest}), (B) Emitted frequency and echo frequency during flight, calculated for targets ahead by using the flight speed (C). The reference frequency corresponds to the averaged echo frequency. The arrow indicates the beginning of the approach phase.

All bats had small variations by up to 30 Hz in f_{rest} and f_{echo} between the flights within one experimental session, which conformed to the standard deviation. Higher variation in f_{rest} and f_{ref} were observed between the days in all bats by up to 90 Hz. However, the correlations of f_{rest} and f_{ref} in bat 1 and bat 2, with regression lines almost parallel to the bisector ($r^2(8) \geq 0.62$, $p \leq 0.0067$) indicate a tight coupling of f_{rest} to f_{ref} (Fig. 5). The f_{echo} of bat 2 was more stable, remaining within the standard deviation. In this bat, f_{echo} varied maximally by 30 Hz between days and f_{rest} only by 20 Hz, if we neglect one outlier of almost 70 Hz.

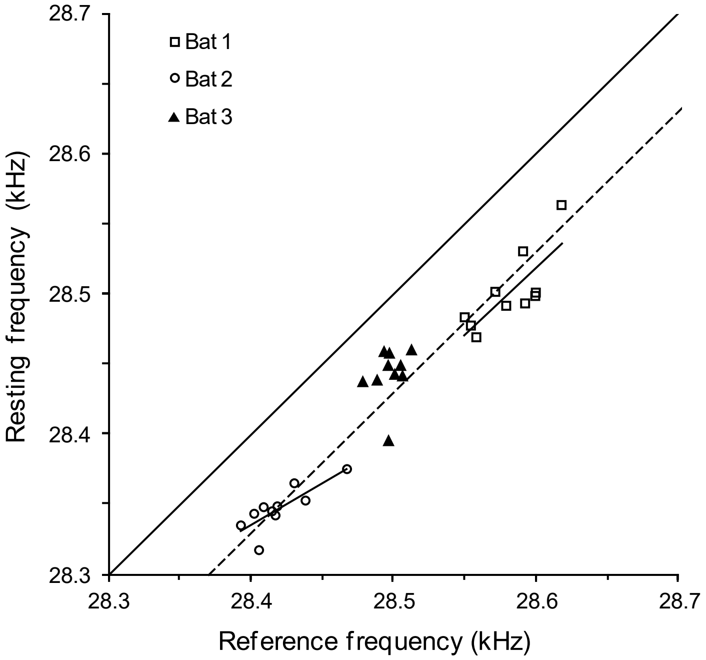

Fig. 5: Coupling between the means of the resting and reference frequencies, with variations over three weeks. For each bat, 10 flights with linear regression lines are shown. The solid line indicates the angle bisector and the dotted line indicates the average offset of 70 Hz.

The precision of the DSC feedback control system of a species is described by the accuracy with which f_{echo} is kept at f_{ref}, independent of the encountered DS and thus independent of flight speed. In *R. paradoxolophus*, f_{echo} was influenced by the flight speed [F(14,27) = 17.90, p < 0.0001]. However, f_{echo} showed no significant changes for moderate flight speeds between 1.75-3.5 m/s (Tukey-Kramer p<0.05 n.s. for flight speeds between 1.75-3.5 m/s). At lower flight speeds (<1.75 m/s), the means tended to be below the f_{ref} with higher standard deviations (Fig. 6).

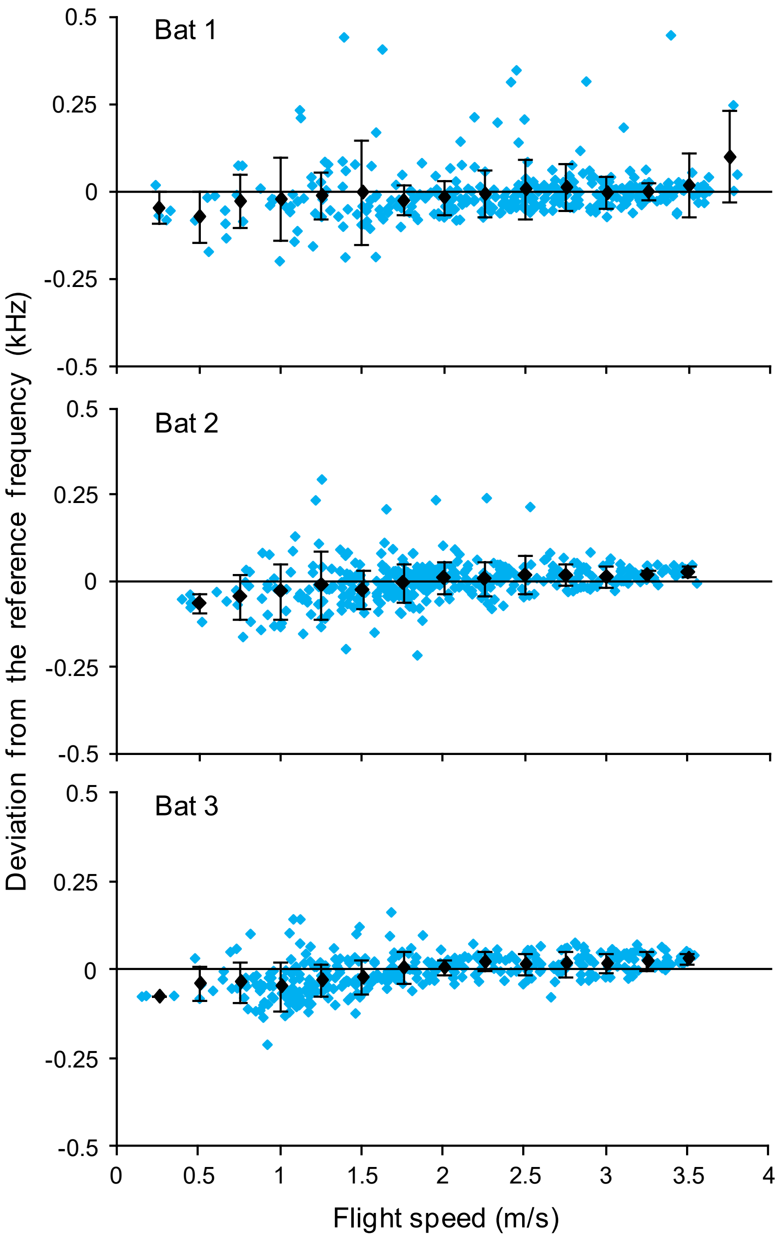

Fig. 6: Precision of Doppler shift compensation behaviour in *R. paradoxolophus*. Deviation of the echo frequency of each call from the reference frequency of the corresponding flight was calculated and plotted against the flight speed. Black diamonds indicate means (±s.d.) calculated for 0.25 m/s classes. Number of flights per bat = 10.

Experiment 2

Behavioural audiogram

The audiogram of bat 1 measured with the Preyer reflex showed a similar course to the audiograms measured in other rhinolophids with a distinct auditory fovea. The audiogram revealed a sharp minimum at 29.3 kHz with a threshold value of 3.8±0.3 dB SPL (±SEM) (Fig. 7). Below the threshold minimum, sensitivity decreased by about 40 dB per octave and above the minimum by about 64 dB per octave. Just below the threshold minimum, at 29 and 26 kHz, the threshold was 35 dB higher. The threshold increase from the minimum towards higher frequencies was less steep than towards lower frequencies. At frequencies slightly higher than the minimum frequency, up to 31.3 kHz, the threshold increased gradually in steps of 0.5-2.7 dB SPL (in total 4.9 dB), followed by a steep increase in frequencies from 32.3 to 36 kHz, where the maximum was reached with 77±4.4 dB SPL (±SEM). At higher frequencies, the threshold level stayed between 50 to 100 dB SPL. The mean f_{rest} measured in this experiment was 29.16 kHz, thus 140 Hz below the threshold minimum. While it should be noted that the behavioural audiogram was measured 12 months earlier than the DSC in the flight experiment, in the same bat the f_{rest} dropped by ~700 Hz from 29.16 kHz to 28.5 kHz indicating a long-term frequency variation.

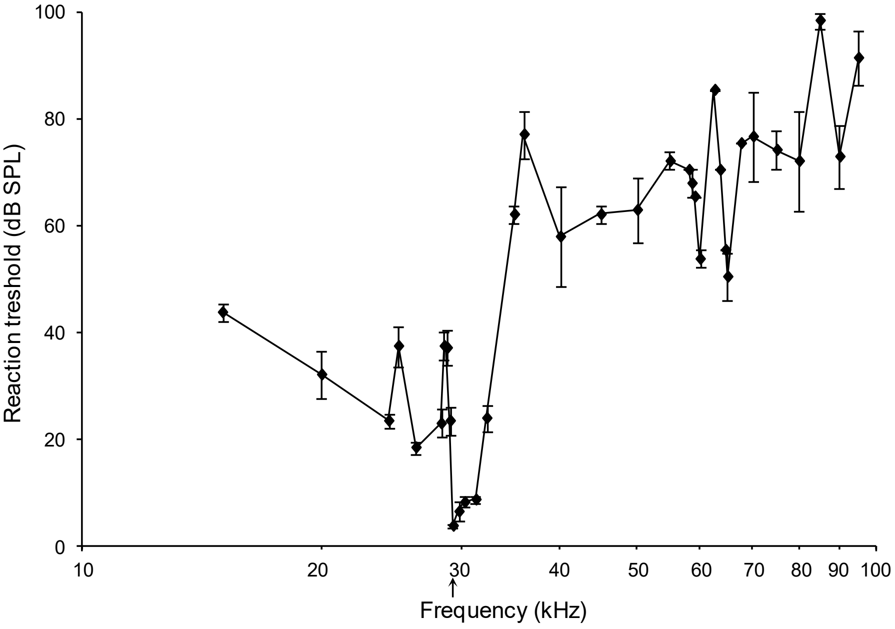

Fig. 7: Behavioural audiogram of *Rhinolophus paradoxolophus* (bat 1) measured with the Preyer reflex (Mean±SEM). The minimum of the audiogram at 29.3 kHz (arrow) was 140 Hz above the resting frequency of the tested individual.

Discussion

The echolocation systems of high duty cycle bats are specialized to evaluate amplitude and frequency modulations in the echoes caused by the moving wings of fluttering insects (Schnitzler et al., 1983; for reviews, see Neuweiler et al., 1980; Schnitzler and Henson, 1980; Schnitzler and Ostwald, 1983). These flutter detecting foragers show specific adaptations of the transmitter and receiver of their echolocation systems to make flutter evaluation possible (Schnitzler and Ostwald, 1983; Schnitzler and Denzinger, 2011). The transmitter is characterized by the emission of echolocation calls with a long constant frequency component and DSC. In flight they adjust the emission frequency in such a way that CF_2 in echoes from stationary targets ahead is kept constant at the average echo frequency f_{ref}, thereby compensating the DS caused by their own flight movements (Schnitzler, 1968). The receiver is characterized by an auditory fovea in the cochlea and a foveal organization of the hearing system. Morphologically, the auditory fovea on the basilar membrane is a highly expanded frequency representation, which is centred to f_{ref} (Schuller and Pollak, 1979; Bruns and Schmieszek, 1980; Vater et al., 1985; Kössl and Vater, 1985; Dannhof and Bruns, 1991; Neuweiler, 1990). The afferent projections from the fovea lead to foveal areas in the auditory pathway with a high overrepresentation of sharply tuned neurons with best frequencies near the f_{ref} (Suga and Jen, 1977; Jen and Suthers, 1982; Rübsamen et al., 1988; Ostwald, 1984). These neurons are very sensitive to flutter information in form of amplitude and frequency modulations contained in the echoes from fluttering insects. The auditory fovea is also mirrored in the audiograms of high duty cycle bats. They show a distinct minimum at f_{ref} and a distinct threshold increase to lower and higher frequencies (reviewed in Schnitzler and Denzinger, 2011).

Studies on the echolocation system of flutter detecting foragers have been limited to a few key species from each family. Within the Rhinolophidae, studies were conducted with *Rhinolophus ferrumequinum*, *Rhinolophus ferrumequinum nippon*, *Rhinolophus euryale* and *Rhinolophus rouxii*. These findings were generalized for all Rhinolophids. *Rhinolophus paradoxolophus*, however, is an outlier in the allometric relationship between body size and CF_2, emitting a much lower CF_2 than expected from body size (Thong, 2011). Whether the adaptations for flutter detection are also found and also match in this low frequency species and whether theses adaptations are as sophisticated as in other rhinolophids were subject of this study.

Echolocation behaviour in flight

Besides the low CF_2 of the echolocation calls, flight and echolocation behaviour of *R. paradoxolophus*, corresponded to that of typical rhinolophids when approaching a target. As other rhinolophids, *R. paradoxolophus* operates in the second harmonic while the first harmonic is strongly and the upper harmonics completely suppressed (Fig. 1). In some studies, it was presumed, that the echolocation signals of *R. paradoxolophus* and *R. rex*, also a member of the *philippinensis* group, have the main energy at the first harmonic (Eger and Fenton, 2003; Huihua et al., 2003; Zhang et al., 2009). However, this was a misinterpretation, presumably because the other harmonics in these calls are often so weak, that they are not visible in the spectrograms. Owing to the low frequency of the second harmonic it was misinterpreted as first harmonic. We are convinced that the functional harmonic of all rhinolophids is the second harmonic.

In orientation flight, *R. paradoxolophus* used 50 ms long single signals at a duty cycle of 50%, which is in accordance to other rhinolophids emitting signal durations of 27-65 ms at duty cycles of 41-57% in orientation flight or when calling from perches (Schnitzler, 1968; Tian and Schnitzler, 1997; Fenton et al., 2012). The increase in bandwidth by *R. paradoxolophus* during approach was smaller than measured in *R. ferrumequinum* (Table 2) (Tian and Schnitzler, 1997). As in *R. ferrumequinum*, the iFM was absent within groups and bandwidth decreased, supporting the conclusion that the iFM is less important or even a by-product of vocalization (Tian and Schnitzler, 1997). Nevertheless, in our study one bat produced an intra-group iFM in groups with a high number of signals for unknown reasons.

In orientation flight, *R. paradoxolophus* emphasized the CF more than the iFM and tFM.

Shortly, before the approach started, the relative rms SPL of the CF and tFM were approximately the same. During approach up to landing, however, the relative rms SPL of the tFM was higher than that of the CF component, indicating that the tFM is used to coordinate landing. The relative rms SPL of the tFM at the microphone was kept constant (slopes -0.7 to 0.4), corresponding to a reduction in emission SPL by 6-7 dB/hd. The reduction of the relative rms SPL of the CF was slightly higher. The reduction of 3-3.8 dB/hd at the microphone corresponded to a decrease in emission SPL by 9-9.8 dB/hd. The relative rms SPL of the iFM was in between that of the CF and tFM. Hiryu et al. (2008) measured in *R. ferrumequinum nippon* via an on-board microphone a reduction of emission SPL of the total signal by 7.6 dB/hd. However, in view of the divergence of the CF/tFM ratio between orientation and approach phase it is essential to analyse the two components, separately, which was only done by Tian and Schnitzler (1997). They measured a reduction of the SPL of the CF by 6-9 dB/hd and a slightly higher reduction of the tFM by 9-11 dB/hd in *R. ferrumequinum*. In *R. paradoxolophus*, we measured the same reduction in the relative rms SPL of the CF, but a smaller reduction in the relative rms SPL of the tFM by 6-7 dB/hd. This may be caused by differences in the target strength of the landing sites, as our bats approached a grid placed at the ceiling, whereas in the study by Tian and Schnitzler (1997) the bats flew to a landing bar. The reduction of the SPL of the tFM in *R. paradoxolophus* also corresponds to the reduction in emission SPL by 6-7 dB/hd in other FM bat species during approach (summarized in Koblitz et al., 2011), which is a further indication that the tFM is used to guide landing.

Doppler shift compensation system and frequency variation

Our study confirmed the findings from other high duty cycle bats that f_{ref} is higher than f_{rest}. This offset between f_{rest} and f_{ref} in *R. paradoxolophus* was with 70 Hz on average quite low, but with 0.25% comparable to that of other flutter detecting foragers (Schnitzler and Denzinger, 2011) (Table 2).

In previous studies, a small offset was assumed to be an indicator for a high quality of DSC. The offset was therefore used by some authors to compare the quality of DSC performance in different species (Habersetzer et al., 1984; Gaioni et al., 1990; Keating et al., 1994). We think that this approach is not constructive, as the best measure to assess the quality of a feedback control system is the precision with which the parameter under control (i.e. f_{echo}) is kept constant at the reference parameter f_{ref}, independent of flight speeds (Schnitzler and Denzinger, 2011; Schoeppler at al., 2018). This has been shown for *R. ferrumequinum* (Schnitzler, 1973) and *Hipposideros armiger* (Schoeppler et al., 2018). Similar to *H. armiger*, we found an influence of flight speed on f_{echo}, however there was no significant effect on f_{echo} at moderate flight speeds of 1.75-3.5 m/s. Due to accelerated and slowed down flight during start and landing, the variation at low fight speeds was higher, which is indicated by the higher standard deviations. The small standard deviation of 60 Hz in f_{ref} corresponded to a variation by 0.21%, which is in the range of the values measured in other rhinolophids, in *P. parnellii* (Schnitzler and Denzinger et al., 2011) and also in hipposiderids (Schoeppler et al., 2018) (Table 2). This shows that the quality of DSC in *R. paradoxolophus* is similar to that of other flutter detecting foragers. Comparable results to other rhinolophids and hipposderids were also found in the ability of *R. paradoxolophus* to keep f_{rest} constant with a deviation of about 30 Hz, which corresponds to 0.11%.

Table 2: Comparative overview of offset, standard deviations, and bandwidth. Data from *Rhinolophus paradoxolophus* (this study), *Rhinolophus ferrumequinum* ([1]Schnitzler, 1968; [2]Schnitzler and Denzinger, 2011; [3]Schuller et al. 1974; [4]Tian and Schnitzler, 1997) and the hipposiderid bat *Hipposideros armiger* ([5]Schoeppler et al., 2018). The percentage values refer to the respective resting frequency (f_{rest}).

	R. paradoxolophus	*R. ferrumequinum*	*H. armiger*[5]
f_{rest}	28.4 kHz	83.0 kHz[1]	65.5 kHz
offset	0.07 kHz (0.25%)	0.15–0.20 kHz[1,2] (0.18–0.24%)	0.08 kHz (0.12%)
SD of f_{rest}	0.03 kHz (0.11%)	0.05 kHz[1] (0.06%)	0.14 kHz (0.21%)
SD of f_{echo}	0.06 kHz (0.21%)	0.03–0.20 kHz[2,3] (0.04–0.24%)	0.11 kHz (0.17%)
tFM orientation flight	2.3 kHz	12.5–15.3 kHz[4]	6.4 kHz
tFM terminal group	3.9 kHz	up to 22 kHz[4]	7.0 kHz
bandwidth increase	1.6 kHz	up to 9.5 kHz	0.6 kHz

In *R. paradoxolophus* the values of the paired f_{rest} and f_{ref} varied over time. We observed small variations of maximally 30 Hz within a recording session, which is in the range of the standard deviation. Variations between days were higher and measured up to 90 Hz. This variation had no impact on the offset, since f_{rest} and f_{ref} were coupled. This coupling was already described in a hipposiderid bat (Schoeppler et al., 2018) and suggested for all flutter detecting foragers. In bat 3, the correlation was not significant, due to less variation in frequency. We assume that the small variations in f_{rest} and f_{ref} were due to the variable physiological parameter body temperature, which was already described for other DS compensating bats like, *P. parnellii* and *H. armiger* (Huffmann and Henson, 1993; Schoeppler et al., 2022). The body temperature most likely changes the mechanical properties of the basilar membrane, which would result in a different stimulation of the foveal area in the cochlea. This activates the audio-vocal feedback control system and leads to a readjustment of the emission frequency.

Several studies mention also individual CF_2 variation in adult rhinolophid bats, in experiments lasting several days up to months. Schuller et al. (1974) found individual variations of f_{rest} by up to 90 Hz in *R. ferrumequinum*, at different days. Hiryu et al. (2008) and Fursawa et al. (2012) described similar variation in *R. ferrumequinum nippon* of 100-260 Hz in f_{rest}.

Besides small variations in frequency, we also observed a large and consistent frequency drop of 700 Hz in bat 1 between the measurement of the auditory threshold and the flight experiments, which were more than 12 months apart. This bat was in a good health condition. The large and consistent change in frequency might be caused by a morphological change of the cochlea by aging processes, similar to the observation in old *R. ferrumequinum* in the field by Jones and Ransome (1993), who found a decrease by up to 1 kHz of f_{rest} in bats older than 10 years, having a good body condition.

Behavioural audiogram

Receiver properties are described by the behavioural and neural audiograms. In all so far studied flutter detecting foragers, they are characterized by a sharply tuned minimum around f_{ref} and a distinct maximum just below (rhinolophids: Neuweiler, 1970; Schnitzler et al., 1971; Long and Schnitzler, 1975; Schnitzler et al., 1976; Schuller, 1980; Henson et al., 1985; Taniguchi, 1985; *P. parnellii*: Grinell, 1970; Pollak et al., 1972; Suga et al., 1975; Henson et al., 1985; hipposiderids: Grinnell and Hagiwara, 1972; Schuller, 1980, Neuweiler et al., 1984; for reviews, see Neuweiler et al., 1980; Schnitzler and Denzinger, 2011). Overall, the behavioural auditory threshold of *R. paradoxolophus* measured with the Preyer reflex in this study strongly resembled the course of these audiograms. We found a sharply tuned threshold minimum at 29.3 kHz, which corresponded to f_{ref} estimated from the coupled f_{rest}, a maximum about 500-750 Hz just below the minimum, a steep rise of the threshold above 31.3 kHz and a sustained high threshold at higher frequencies. The steep increase in the auditory threshold of almost 40 dB SPL just below the minimum covers the frequency range of the emitted CF_2 during flight, i.e. at flight speeds up to 3.8 m/s, the CF_2 is 625 Hz below f_{ref}. This maximum is characteristic for all DSC bats and is considered as prevention to vocal self-stimulation (Grinnell, 1970; Neuweiler, 1970; Suga et al., 1975). A second maximum around the first harmonic found in other rhinolophids and hipposiderids was also obvious in *R. paradoxolophus* around 15 kHz. In between these two maxima, there was another broader sensitive region, also reported in audiograms of *R. ferrumequinum*, *R. ferrumequinum nippon* and *R. rouxi* (Neuweiler, 1970; Neuweiler et al., 1971; Long and Schnitzler, 1975; Taniguchi, 1985; Kössl, 1994). In *R. ferrumequinum*, this broadly expanded minimum is located around 60 kHz and allocates to the FM component, which has bandwidths of 13-22 kHz (Long and Schnitzler, 1975; Tian and Schnitzler, 1997). In *R. paradoxolophus*, the broader sensitive region had its minimum just 3 kHz below the f_{ref}, at 26.3 kHz. This corresponded exactly to the FM, which had a bandwidth of 2-4 kHz.

In rhinolophids, there is another minimum in frequencies around 15-30 kHz, considered to be important for the processing of communication signals (Long and Schnitzler, 1975). Due to technical constraints, we could not measure thresholds lower than 15 kHz in *R. paradoxolophus.* We therefore cannot exclude that the hearing threshold may get more sensitive in frequencies in the range of the communication signals, which have the highest amplitude below 10 kHz (D.S., personal observation).

Above the minimum at f_{ref}, the auditory threshold of *R. paradoxolophus* increased in steps of 0.5-2.7 dB SPL up to a frequency of 31.3 kHz, followed by a steep rise. In higher frequencies of 35-95 kHz, the thresholds remained high with 70±12 dB SPL on average, and we did not find another threshold lowering in the range of higher harmonics. The audiograms measured in previous studies did not include frequencies around higher harmonics. Overall, the threshold minimum at f_{ref} in the behavioural audiogram and the adjustment of f_{echo} at f_{ref} by DSC documents a tight match between transmitter and receiver properties.

Does the match of transmitter and receiver properties in rhinolophids support the harmonic hopping hypothesis?

Within the family of the Rhinolophidae, bats of the *philippinensis* group, to which also *R. paradoxolophus* belongs, form a paraphyletic clade comprising 11 species (Zhang et al., 2018). They all have in common that they emit CF_2 lower than predicted from allometry (Francis and Habersetzer, 1998, Zhang et al., 2018). Kingston and Rossiter (2004) studied the three distinct, sympatric size morphs of *R. philippinensis*, also members of the *philippinensis* group and showed that the CF_2 of the smaller morphs of *R. philippinensis* corresponds to higher harmonics of the fundamental of the largest morph, with the large morph being the ancestral form. They argue that shifting to another harmonic provides new ecological opportunities and may result, mediated by the communication function of echolocation calls, in assortative mating, which finally leads to reproductive isolation and speciation. They conclude that the rapid radiation of rhinolophids in South East Asia may be facilitated by harmonic hopping.

We doubt that the proposed harmonic hopping mechanism explains the speciation in rhinolophids of the *philippinensis* group. The harmonic hopping argument is based alone on variations of transmitter properties. But flutter detection needs corresponding adaptations of the auditory receiver. DSC delivers the flutter information in echoes with a carrier frequency near f_{ref}, which is the frequency range where the auditory fovea of a highly specialized hearing system is a prerequisite for the decoding of this information. Flutter detecting works only at a close match of transmitter and receiver adaptations. If we assume that an ancestral low frequency rhinolophid would hop upwards to echolocation calls with frequencies around the third or the fourth harmonic, the echoes would impinge on a high auditory threshold above the auditory fovea, where the hearing system is not equipped for flutter detection. Downward hopping with a frequency shift from the second to the first harmonic would also not work, as the f_{echo} would fall into the range of a threshold maximum, found in all so far studied high duty cycle bats. The tight match between transmitter and receiver properties in flutter detecting foragers makes it evident that a change in transmitter properties through harmonic hopping would require a corresponding jump of the morphologically defined auditory fovea and its afferent areas in the brain. A jump of one octave in bats having a hard-wired specialized auditory fovea is beyond comprehension as brains don't hop.

Conclusions

This study proves for *R. paradoxolophus*, an allometric outlier with a low frequency echolocation system, a tight match between transmitter and receiver properties. During DSC, *R. paradoxolophus* adjusted the echo frequency with high precision at f_{ref}, and the minimum of the behavioural audiogram, which indicates the highest sensitivity of the foveal hearing system, matched with f_{ref} estimated from the coupled f_{rest}. Our result supports the current view on the function of the audio-vocal control system for DSC (Schoeppler et al., 2018; 2022). The reference frequency (f_{ref}), which is the controlled process variable of the audio-vocal control system in flutter detecting foragers is not a fixed frequency value, but is determined by the activation state of the morphologically defined foveal resonance area in the cochlea and in the connected higher foveal centers of the hearing and the vocal control system. DS compensating bats readjust the emission frequency and with it f_{ref}, if the cochleotopic input is changed and the reported activation state of the foveal area differs from the set point values of the vocal control system. A change of the resonance frequency of the fovea by changes in body temperature or by long term morphological changes, e.g., through aging, will lead to a concomitant change in f_{ref}. The shift of f_{ref} in bat 1 by 700 Hz over 12 months suggests such a change of the resonance properties in the cochlea.

Species-specific echolocation frequencies, especially the low frequencies of *R. paradoxolophus*, require morphological adaptations of all functional parts of the transmitter and receiver system to the corresponding CF_2. The wave-length dependent structures of the nose-leaf, the pinna, the resonance chambers in the vocal tract and of the vocal cords, and also the frequency dependent position and structure of the foveal resonance area in the cochlea must be adjusted to the species-specific CF_2. Overall, our results support the assumption that the echolocation systems of all flutter detecting foragers possess similar highly adapted transmitter and receiver systems for flutter evaluation.

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Supplementary Information

Fig. S1

Fig. S2

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Supplementary information of chapter 2

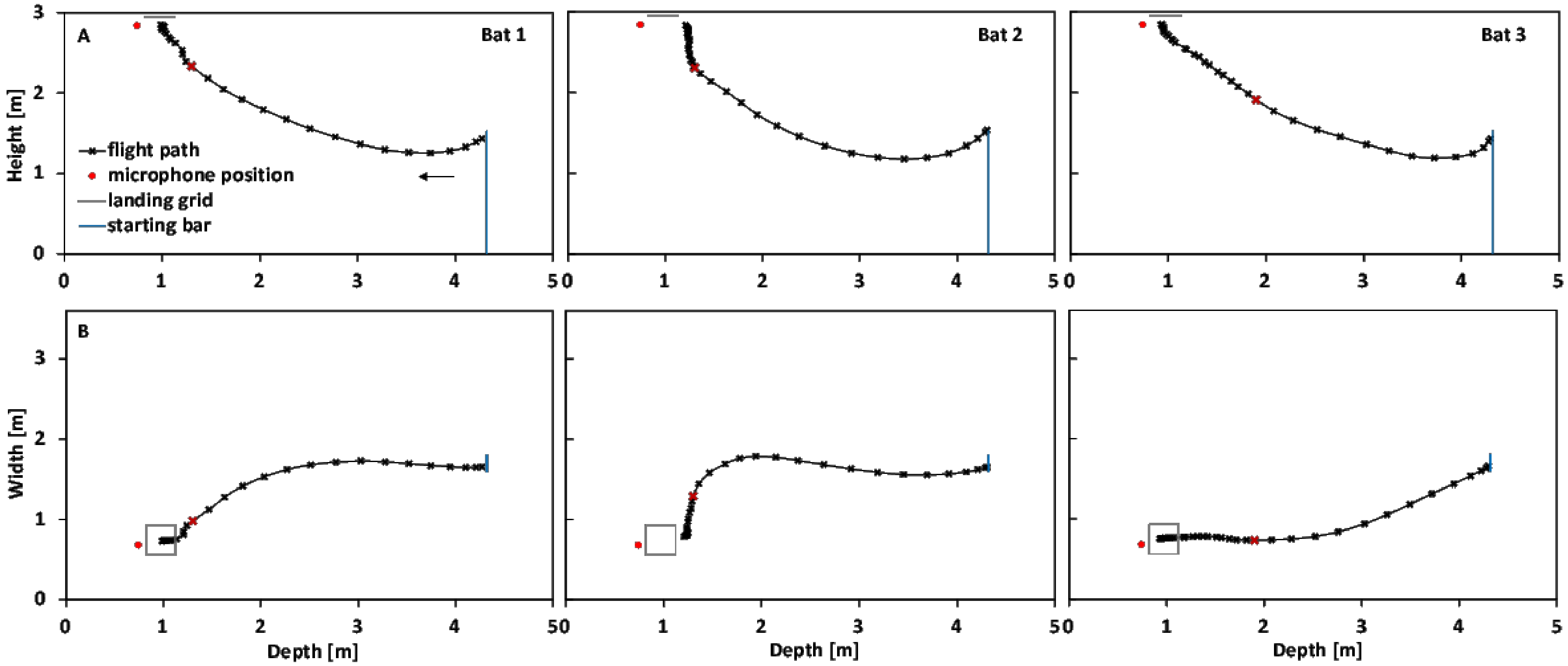

Fig. S1: Flight paths of three *Rhinolophus paradoxolophus* flying to a landing grid. The lateral view is depicted in the top row (A), the top view in the lower row (B). The grey square indicates the landing grid and the arrow the flight direction. The positions of signal emission are marked with a cross, while the cross in red indicates the beginning of the approach.

Supplementary information of chapter 2

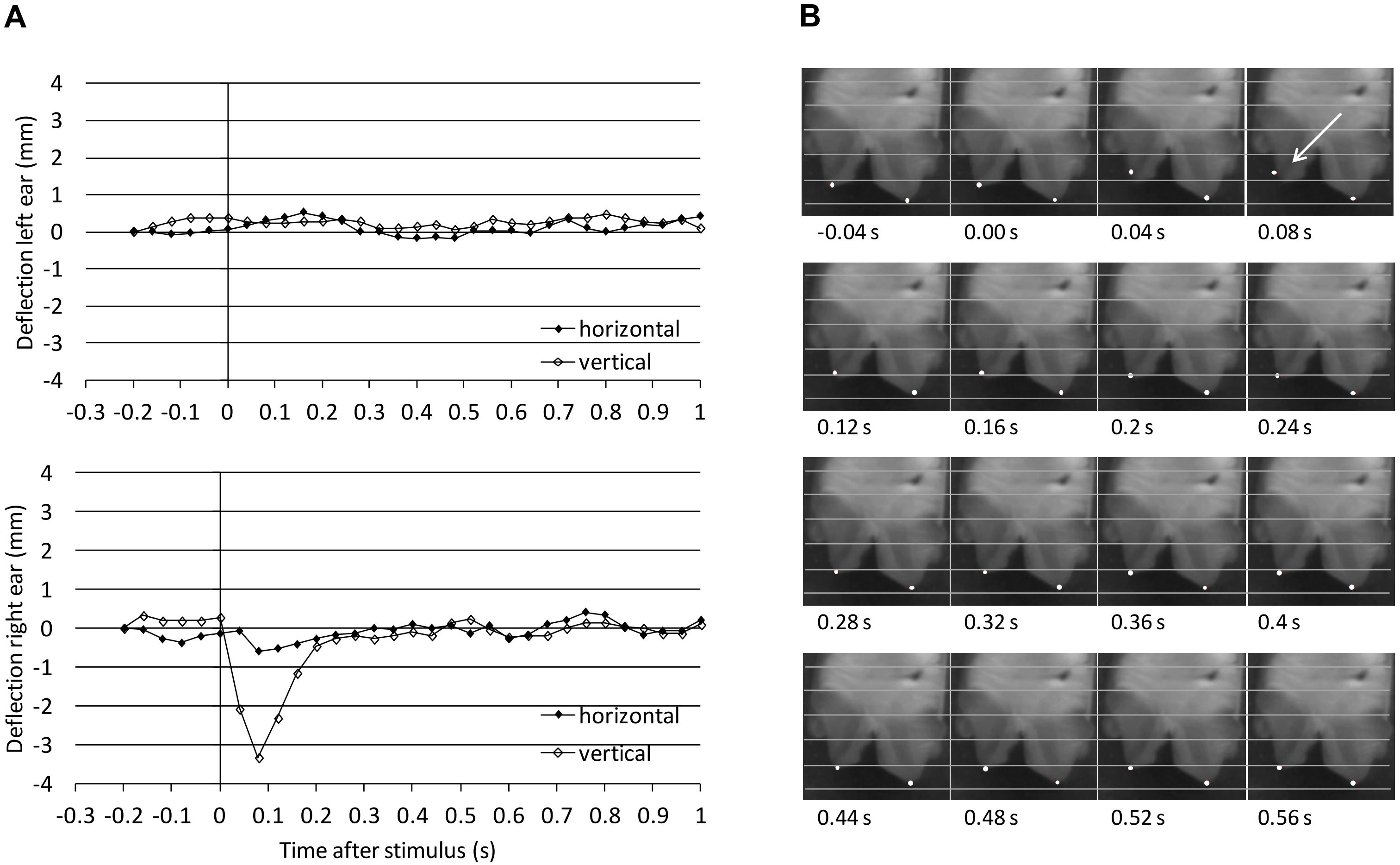

Fig Fig. S2: Ear movement of *R. paradoxolophus* as reaction of a pure tone stimulus of 55 kHz at 70.5 dB SPL. The graphs display the horizontal and vertical deflection of the left and right pinna (A). The stimulus was presented at time 0. Frames of the video recording (B) show the reaction 40 ms before and 560 ms after the stimulus representation (shaded area in A). Tips of the pinnae are marked with a white dot. The arrow indicates the reaction.

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Curriculum Vitae

To ensure data privacy, this part is not available in the online version.