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## Evolution of a Communication System by Sensory Exploitation of Startle Behavior

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2

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17

18

19 **SUMMARY**

20 New communication signals can evolve by sensory exploitation if signaling taps into pre-existing  
21 sensory biases in receivers [1, 2]. For mate attraction, signals are typically similar to attractive  
22 environmental cues like food [3-6], which amplifies their attractiveness to mates, as opposed to  
23 aversive stimuli like predator cues. Female field crickets approach the low-frequency calling song  
24 of males, whereas they avoid high-frequency sounds like predatory bat calls [7]. In one group of  
25 crickets (Eneopterinae: Lebinthini), however, males produce exceptionally high-frequency calling  
26 songs in the range of bat calls [8], a surprising signal in the context of mate attraction. We found  
27 that female lebinthines, instead of approaching singing males, produce vibrational responses after  
28 male calls, and males track the source of vibrations to find females. We also demonstrate that field  
29 cricket species closely related to the Lebinthini show an acoustic startle response to high-frequency  
30 sounds that generates substrate vibrations similar to those produced by female lebinthine crickets.  
31 Therefore, the startle response is the most likely evolutionary origin of the female lebinthine  
32 vibrational signal. In field crickets, the brain receives activity from two auditory interneurons;  
33 AN1 tuned to male calling song controls positive phonotaxis, and AN2 tuned to high-frequency  
34 bat calls triggers negative phonotaxis [9, 10]. In lebinthine crickets, however, we found that  
35 auditory ascending neurons are only tuned to high-frequency sounds, and their tuning matches the  
36 thresholds for female vibrational signals. Our results demonstrate how sensory exploitation of anti-  
37 predator behavior can evolve into a communication system that benefits both senders and  
38 receivers.

39

## 40 **RESULTS**

41 Animals generate signals using a variety of modalities to influence the behavior of receivers, and  
42 various mechanisms have been proposed for the evolution of these diverse signals [11]. In some  
43 cases, novel signals evolve because they tap into a pre-existing sensory bias and associated  
44 behavioral response in the receiver that is beneficial to the sender of the signal, a process called  
45 sensory exploitation [1, 2]. In almost all known cases, mating signals that arise through sensory  
46 exploitation capitalize on attractive stimuli, like food cues [3-6] or refuges from predators [12],  
47 which increases the likelihood of gaining the receiver's attention and motivating the receiver to  
48 approach. In the context of mate attraction, signals that tap into pre-existing sensory specializations  
49 for predator defense are, understandably, extremely rare (but see [13]). An important step in  
50 determining whether a signal arose through sensory exploitation is demonstrating that the response  
51 to the signal preceded the appearance of the signal itself, a step that requires a phylogenetic  
52 framework, which is not always available [2]. Furthermore, we seldom understand the neural  
53 origins of sensory biases, despite the important role that neural tuning and sensitivity plays in this  
54 process (but see [14]).

55 In this study, we combine behavioral and neural data in a phylogenetic context to assess the  
56 role of sensory exploitation in the evolution of an unusual communication signal in crickets.  
57 Female field crickets (Gryllidae: Gryllinae) approach low-frequency sounds typical of male calling  
58 song (~5 kHz) and avoid high-frequency sounds (>10 kHz) like the echolocation calls of predatory  
59 bats [7, 15]. Two ascending auditory interneurons, AN1 and AN2, forward activity from auditory  
60 afferents in the thorax to the brain [9]. AN1 is narrowly tuned to the low frequencies of the calling  
61 song, and it initiates positive phonotaxis [16]. AN2 is tuned to higher sound frequencies and  
62 triggers avoidance behavior in response to bat calls [10]. Eneopterine males (Gryllidae:

63 Eneopterinae) are the only crickets known to generate calling songs with intense high-frequency  
64 harmonics [8]. In one derived tribe, the Lebinthini, one of the higher-frequency harmonics has  
65 become the dominant frequency of the call, and this harmonic is ultrasonic ( $>20$  kHz) in some  
66 species [8]. Considering that in closely related field cricket species [17], high-frequency sounds  
67 provoke avoidance behavior [7, 18], the use of high-frequency calling songs for mate pairing is  
68 surprising.

69 We considered two hypotheses for the origin of high-frequency calling songs in lebinthine  
70 crickets. Most of the energy in male field crickets calls is restricted to a low frequency band, but  
71 there is still significant energy in higher frequency harmonics [19]. Although these harmonics are  
72 not required for phonotaxis, female crickets of at least one species prefer and can more accurately  
73 locate song models when they contain these higher frequency harmonics [20]. Eneopterine crickets  
74 often live on plants instead of on the ground, and increasing the energy in the higher-frequency  
75 harmonics of calls might have provided a greater benefit to crickets locating mates in the three-  
76 dimensional habitat of plants, compared to the less complex two-dimensional habitat of crickets  
77 that live on the ground. The high-frequency calls of lebinthine crickets could have been a result of  
78 selection for a call feature more suitable for their complex environment. This hypothesis predicts  
79 that female lebinthine crickets will preferentially select males with higher frequency calls. High-  
80 frequency calls could have also arisen by sensory exploitation if males were exploiting an anti-  
81 predator behavior in females. This second hypothesis predicts that female lebinthine crickets  
82 demonstrate anti-predator-like behavior in response to male calls.

83

84 **Female cricket behavior in response to male calling song**

85 To test these hypotheses, we first analyzed and compared female behavior during male calling  
86 songs in five cricket species within the family Gryllidae (Figure 1). Two species have low-  
87 frequency calling songs: *Gryllus bimaculatus*, an outgroup species from the subfamily Gryllinae,  
88 and *Nisitrus vittatus*, from the tribe Nisitrini within the Eneopterinae. Three species have high-  
89 frequency songs: *Cardiodactylus muria*, *Agotecous obscurus*, and *Lebinthus luae*, all from the  
90 tribe Lebinthini within the Eneopterinae. Positive phonotaxis to the male calling song is well  
91 documented in field crickets (Gryllinae) [7, 15]. In arena experiments, we found that female *N.*  
92 *vittatus* also demonstrated a positive phonotactic response by walking to a speaker broadcasting  
93 the male calling song (Figure 2). Females of the three lebinthine species, however, did not walk at  
94 all when presented with the species-specific calling song (Figure 2). Instead, they remained  
95 stationary during male calling song but produced a vibrational response by jerking their body after  
96 each male call. We also observed female lebinthine crickets producing vibrational responses to  
97 male calls in the wild while remaining perched on leaves (Movie S1). We never observed  
98 vibrational responses in *N. vittatus* during playbacks of male calling song (Figure 2).

99 To determine how male and female lebinthine crickets find each other, we made observations  
100 of couples (one male and one female) for two species (*C. muria* and *A. obscurus*) in a cylindrical  
101 mesh arena. Observation sessions were conducted under red light and lasted for 20 minutes from  
102 the time that the male started calling. For *C. muria*, 10 of 13 couples tested located each other, and  
103 for *A. obscurus*, 3 of 5 couples located each other. In all cases, the behavior of males and females  
104 were consistent. The male lebinthine crickets produced calls and then walked a short distance in  
105 the arena before producing another call. Females did not walk, but remained stationary and  
106 produced vibrational signals. For communicating couples, it was always the calling male that  
107 walked to the stationary female. This is different from other species of crickets in which males

108 remain stationary while calling and are approached by females [20-24]. We also observed male  
109 lebinthine crickets in the wild alternating between singing and walking and eventually tracking  
110 down a nearby stationary female producing vibrational signals in response to his calls (Movies S2,  
111 S3).

112 We then assessed the frequency tuning and timing of the female response relative to the male  
113 call. When song models that contained only one of the harmonics of the male call (6, 12, or 18  
114 kHz) were broadcast, females of the low-frequency eneoapterine species *N. vittatus* only showed  
115 phonotaxis to the 6 kHz song model, but not to the 12 or 18 kHz song models (arena tests; N=2).  
116 The vibrational signals produced by female lebinthine crickets in response to computer generated  
117 male calls were broadly tuned to high frequencies (10-20 kHz; Figure 2) and the tuning was similar  
118 to that of the AN2 neuron and negative phonotaxis in *G. bimaculatus* and other field cricket species  
119 [26-28]. The females responded within a narrow range of latencies after the end of each male call,  
120 and the latency differed between species (Table 1).

121

## 122 **The acoustic startle reflex in crickets**

123 We hypothesized that the origin of the female vibrational response could be a startle reflex, a rapid  
124 jerking and freezing movement in response to a sudden stimulus, that is ubiquitous in many  
125 animals [29] including insects [30, 31]. To test if the acoustic startle response preceded the  
126 appearance of the vibrational reply, a condition required for sensory exploitation, we measured the  
127 behavioral responses of four species of field crickets (Gryllinae) and two species of eneoapterines  
128 to a high-amplitude (90 dB SPL) sound pulse at two frequencies: 6 kHz (similar to the dominant  
129 frequency in the calls of most field crickets) and 14 kHz (similar to the dominant frequency in the  
130 calls of many lebinthine species) (Table 1). All four field cricket species reliably generated short-

131 latency substrate vibrations in response to the 14 kHz stimulus, indicating an acoustic startle reflex  
132 (ASR), but never in response to the 6 kHz stimulus. The latencies between the onset of sound and  
133 the onset of the ASR in these four species are similar to the latencies recorded for the bat-avoidance  
134 response by flying crickets (35-66 ms) [32], a behavior known to be triggered by AN2 [10]. In  
135 contrast, the lebinthine species *C. muria* did not show any vibrational response (ASR) to these  
136 sound stimuli (Table 1); instead, tests using different durations and repetition rates of high-  
137 frequency sound pulses indicate that they require the species-specific male calling pattern to  
138 produce a vibrational reply (Figure S1). The latencies for the vibrational reply in lebinthine species  
139 are also considerably longer than what is typical for acoustic startle (Table 1).

140

#### 141 **The neural basis for mate pairing behavior in lebinthine crickets**

142 If the female behavior evolved from a startle response, we would expect the lebinthine AN2 to  
143 have the same frequency tuning as the female vibrational reply. We recorded the spike activity of  
144 ascending auditory neurons (ANs) in the lebinthine *C. muria* and in the field cricket *G. bimaculatus*  
145 (Figure 3). Extracellular recordings from the connectives between the prothoracic and the  
146 subesophageal ganglia in *G. bimaculatus* showed similar activity levels in response to 5 and 14  
147 kHz sound pulses due to AN1 and AN2 activity, respectively (Figure 3A). Connective recordings  
148 in the lebinthine species, however, showed no neural activity in response to 5 kHz pulses and a  
149 high level of activity in response to 14 kHz pulses (Figure 3A). Therefore, there is no low-  
150 frequency sound response forwarded to the brain in *C. muria* that corresponds with the narrow  
151 AN1 tuning known from field crickets [9].

152 Intracellular recordings and staining experiments were conducted to reveal the tuning of  
153 individual auditory interneurons in the brains of these two cricket species (Figure 3B, Figure S2,



154 Figure S3). In *G. bimaculatus*, we could unambiguously discriminate between AN1 (N=20) and  
155 AN2 (N=20) based on their characteristic frequency tuning and morphology. All ascending (N=11)  
156 and local (N=6) auditory interneurons recorded in 11 *C. muria*, however, were only sensitive to  
157 high-frequency sounds with a threshold curve similar to AN2 of *G. bimaculatus*. The threshold  
158 curves of ANs in *C. muria* were consistent between animals and closely matched the threshold-  
159 tuning curve for the female vibrational response (Figure 3B). Thus, both the extracellular and  
160 intracellular recordings demonstrate that the ascending auditory neurons in *C. muria* only respond  
161 to high-frequency sounds. Successful intracellular stainings of ANs in the brains of four *C. muria*  
162 did not show a clear AN1-like or AN2-like morphology as in *Gryllus* (Figure 3C). Therefore we  
163 speculate that either the AN1 has been lost in the lebinthine species or its frequency tuning has  
164 shifted to that of the AN2.

165

## 166 **DISCUSSION**

167 Our results support the hypothesis that sensory exploitation in the communication system of  
168 lebinthine crickets led to the transformation of a startle response to a mate-pairing signal (Figure  
169 S4). This hypothesis suggests that, starting with mate pairing typical for field crickets in which  
170 females approach singing males, the high-frequency components of the calling songs in  
171 eneopterine crickets increased over evolutionary time until the male song triggered an ASR in  
172 females of the ancestral lebinthine crickets. Male lebinthine crickets that followed substrate  
173 vibrations generated by the female had the advantage of finding mates without waiting to be  
174 selected by one and possibly reducing their risk from acoustically orienting predators and  
175 parasitoids [33, 34]. Likewise, females potentially reduced their risk of predation by remaining  
176 stationary. Potential costs to lebinthine females producing ASRs, such as attracting vibration-

177 sensing predators [35], might have selected for females that responded to a narrower range of high-  
178 frequency stimuli (i.e. only the calls of conspecific males) as opposed to any high-frequency sound.  
179 As the vibrational response was co-opted into a true communication signal, individuals of  
180 lebinthine species lost the typical cricket ASR to any high-frequency sound pulse. Thus, what  
181 started as sensory exploitation by males of a startle reflex (ASR) in females evolved into a new  
182 auditory-vibratory communication system with females selectively producing vibrational signals  
183 in response to the calls of conspecific males.

184 The behavior seen in lebinthine females and the presence of phonotaxis in ancestral taxa  
185 suggests that female phonotaxis has been lost in the Lebinthini. Female phonotaxis to male calls  
186 has been documented in three of seven cricket families, including one of the most basal groups  
187 (the Gryllotalpidae) and three subfamilies within the family Gryllidae (Fig. 1A). Because of the  
188 lack of knowledge about the character state in some families, it is difficult to assess if phonotaxis  
189 is the ancestral condition for all crickets. However, the most parsimonious explanation for the taxa  
190 included in this study (Gryllinae and Eneopterinae) is that the ancestral condition of female  
191 phonotaxis was lost once in the ancestor of the Lebinthini, as opposed to female phonotaxis  
192 evolving twice independently in the Gryllinae and Nisitriini.

193 The behavioral pattern seen in female lebinthine crickets is typical of insects that communicate  
194 with acoustic duets in which the male call is followed by a female reply in a species-specific time  
195 window [36]. In lebinthines, however, males and females use different modalities and  
196 communication channels (acoustic and vibrational signals, respectively). Vibration-based  
197 communication through plant substrates is widespread across many insect groups, including the  
198 Orthoptera [37]. The dominant frequencies of the vibrational reply produced by lebinthine crickets  
199 (*C. muria*:  $38 \pm 3$  Hz; *A. obscurus*:  $48 \pm 5$  Hz; *L. luae*:  $84 \pm 4$  Hz; mean  $\pm$  SEM) are in the range

200 of those produced by non-acoustic crickets for vibrational communication [38] and activate the  
201 vibration-sensitive subgenual organ in the legs of crickets [39]. Furthermore, plants transmit  
202 vibrations very well compared to the ground substrate of field crickets [37]. Therefore, these  
203 vibrational signals are well suited for detection by eneopterine crickets.

204 Females of some field cricket species can more accurately locate male song that includes high-  
205 frequency harmonics [20], and this could have selected for greater energy in higher frequency  
206 harmonics in eneopterines due to their tendency to live on the more complex environment of plants  
207 rather than the ground. Because vibratory cues can accompany male singing [40] and courtship  
208 behavior [41] in crickets, and male field crickets are known to phonotactically approach other  
209 singing males [42], territoriality or competition might have been the original impetus for males to  
210 approach the vibrations produced by startled females.

211 The function of the high-frequency ASR in perched insects is unknown because it provides no  
212 protection against attacking bats. It is possible that it is a by-product of the bat avoidance response  
213 during flight and has no adaptive function or significant cost in this context. Both the ASR in  
214 crickets and vibrational signal in female lebinthines are most likely mediated by activity in the  
215 AN2 interneuron, which triggers cricket avoidance steering in flight. The latencies of the  
216 vibrational reply in female lebinthines, however, are significantly longer than what is observed for  
217 ASRs (Table 1), and the vibrational replies are tuned to the temporal pattern of the species-specific  
218 male call, as is seen in other duetting insects [36]. As AN1 is crucial for calling song pattern  
219 recognition in field crickets [43, 44], its tuning in lebinthine crickets might have shifted to higher  
220 frequencies to support pattern recognition. Further experiments are needed on the tuning,  
221 morphology, and targets of the auditory ANs and local brain neurons across eneopterine cricket

222 species with established phylogenetic relationships to assess in more detail how the function of  
223 these neurons changed over evolutionary time in the eneoapterine clade.

224 The origins of communication signals have long fascinated evolutionary biologists, and  
225 multiple potential mechanisms for these origins have been proposed [11, 45]. Evidence from many  
226 well-documented cases over the past 25 years suggest that sensory biases in receivers is a relatively  
227 common origin for novel communication signals [3-6, 12]. In almost all cases of exploitation of  
228 sensory biases for mate finding, senders converge on properties of environmental cues that are  
229 attractive to receivers due to the clear benefits this has for the sender. A particularly interesting  
230 aspect of the lebinthine communication system is that, unlike other examples in which senders  
231 increase the probability that receivers will move towards them, male lebinthine crickets induced a  
232 response in females (the ASR) that then evolved into a new communication signal itself (the  
233 vibrational reply). Our phylogenetic, behavioral, and neurophysiological evidence demonstrates  
234 that sensory exploitation of an aversive cue can also evolve into a classic communication system  
235 in which both senders and receivers benefit, and that this unexpected origin might be more  
236 common than previously appreciated.

237

## 238 **SUPPLEMENTAL INFORMATION**

239 Supplemental Information includes Supplemental Data (4 figures and 3 movies) and Supplemental  
240 Experimental Procedures.

241

## 242 **AUTHOR CONTRIBUTIONS**

243 HtH conducted behavioral and extracellular neural recording experiments. SS conducted  
244 intracellular recording and staining experiments. TR observed and recorded videos of behavior in  
245 the field. All authors contributed to the conception, design and writing of this study.

246

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266

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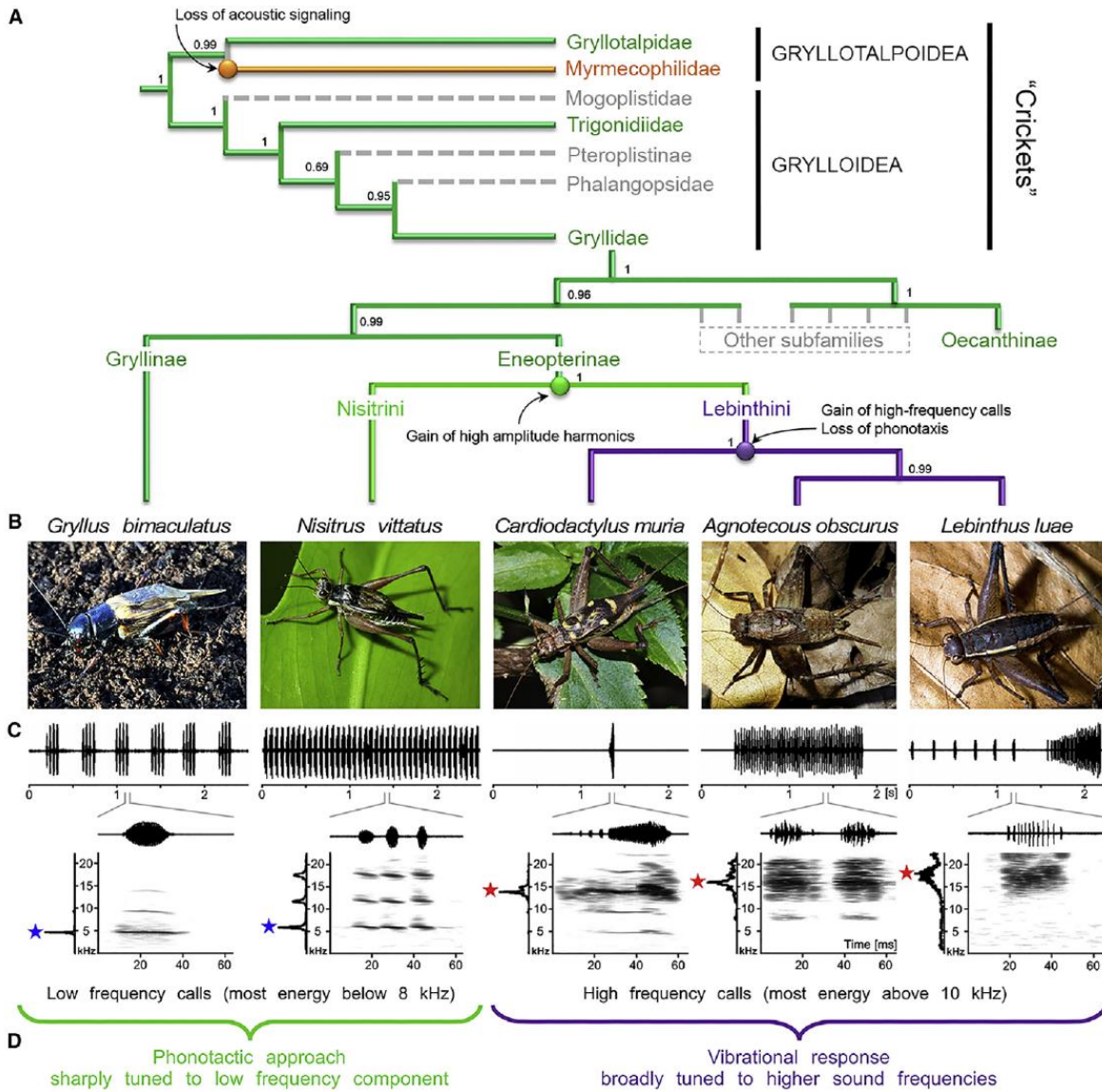
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382 **Figure 1. Phylogenetic relationships, male calling song features, and female behavior in five**  
 383 **cricket species.**

384 (A) Inferred phylogenetic tree of cricket families and subfamilies with a simplified topology,  
 385 based on a large-scale analysis (205 species) using seven nuclear and mitochondrial molecular  
 386 markers [17] showing Bayesian posterior probabilities corresponding to each node. Families and

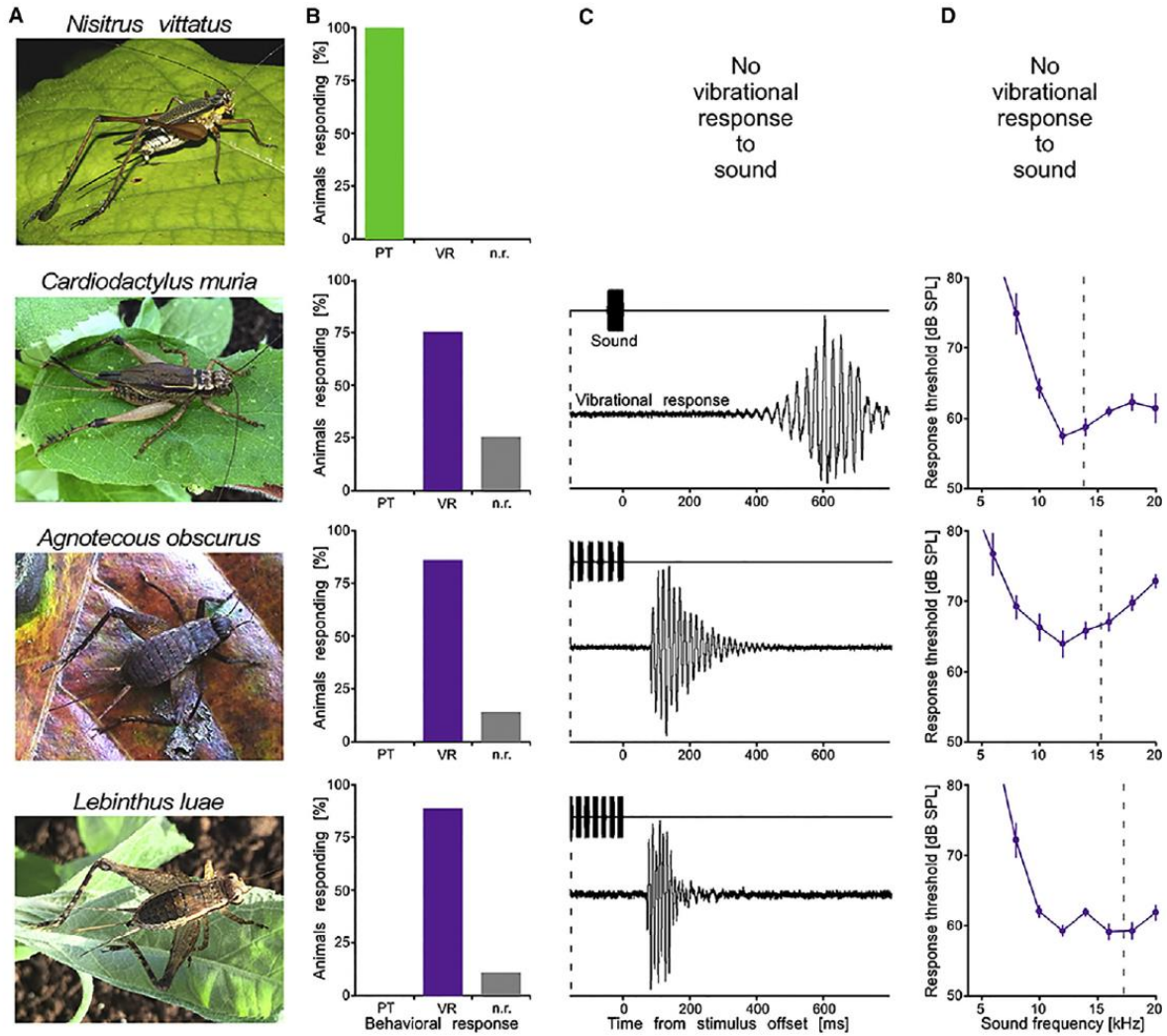
387 subfamilies in which female phonotaxis to male calls has been documented are given in green  
388 [20-24; this study], families and subfamilies in which it is unknown if females perform  
389 phonotaxis to male calls are given in grey with dashed lines, families in which acoustic  
390 communication has been lost are given in orange [25], and the subfamilies which have acoustic  
391 communication but lack phonotaxis are given in magenta [this study].

392 (B) Cricket species investigated in this study and photographs of males.

393 (C) Oscillograms of complete male calling song (upper panel) and oscillograms of individual  
394 sound pulses (middle panel) with power spectra (left lower panel) and spectrograms (right lower  
395 panel) for each species. Stars highlight the dominant frequency of the call.

396 (D) Category of female behavioral responses (phonotaxis or vibrational response) to conspecific  
397 male calling song. See Movies S1, S2, and S3 for examples of the lebinthine male calling and  
398 searching behavior and female vibrational replies.

399



400

401 **Figure 2. Female behavioral responses to male calls in four cricket species.**

402 (A) Cricket species investigated in this study and photographs of females.

403 (B) Behavioral responses of female eneopterines to a speaker broadcasting conspecific male  
 404 calling song; PT: phonotaxis, VR: vibrational response, n.r.: no response (*N. vittatus*, N = 4; *C.*

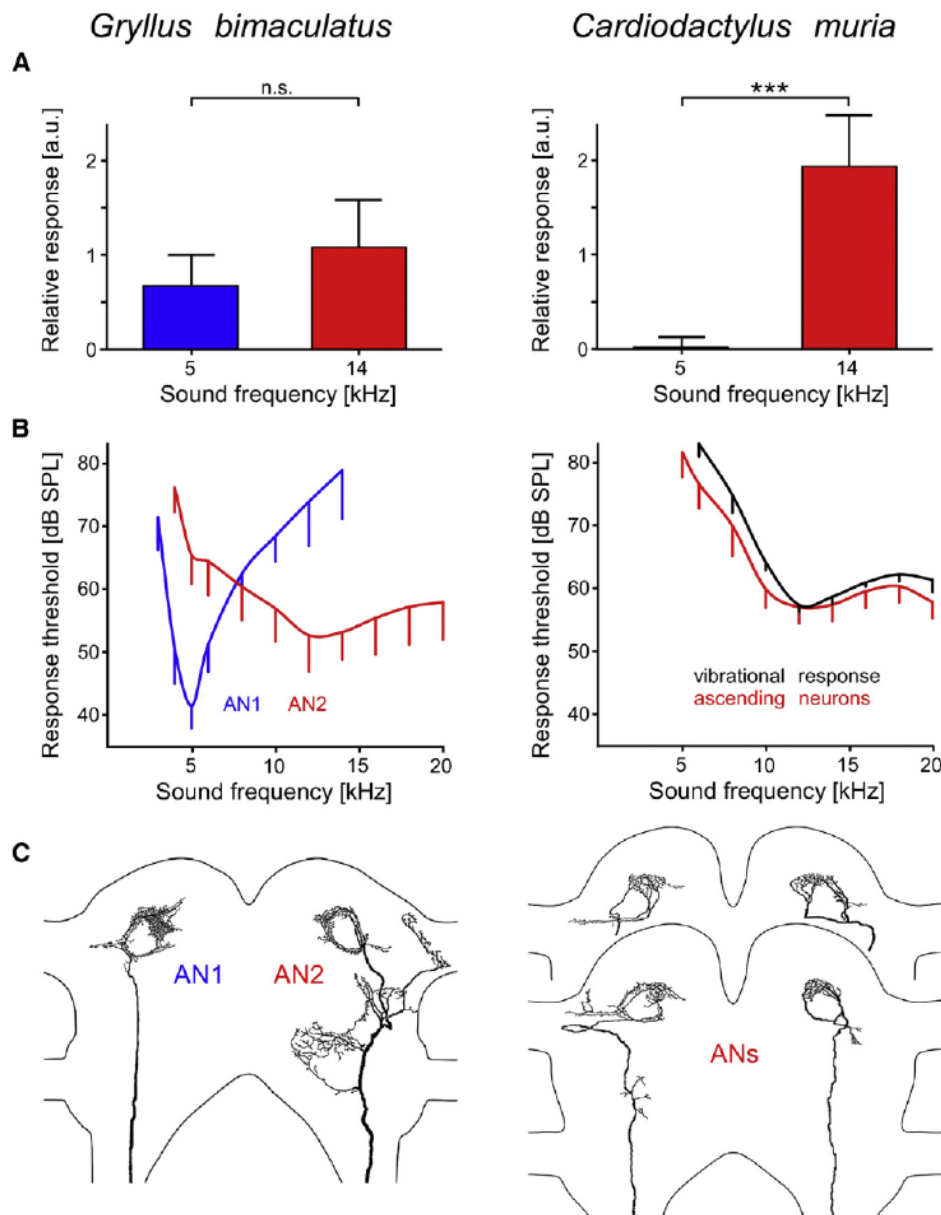
405 *muria*, N = 8; *A. obscurus*, N = 7; *L. luae*, N = 9). The number of individuals producing each

406 type of response was significantly different between species (Chi-squared test using Monte Carlo

407 simulations, 1000 simulations,  $\chi^2 = 28.7$ ,  $p = 0.002$ ).

408 (C) Examples of accelerometer recordings of vibrational replies (lower traces) to male call

409 models (last 150 ms of male call in upper traces) in three lebinthine species.  
410 (*D*) Threshold tuning curves for vibrational replies in each species (mean  $\pm$  SD); dotted lines  
411 indicate the mean peak frequency of male calling song. See also Figure S1 for responses of *C.*  
412 *muria* to sounds of different durations and repetition rates. See Supplemental Experimental  
413 Procedures for methods used to measure threshold response sound levels.  
414  
415



416

417 **Figure 3. Neural response of ascending interneurons to sound for a field cricket (*G.***

418 ***bimaculatus*) and a lebinthine species (*C. muria*).**

419 (A) Standardized relative neural activity in response to sound measured by extracellular

420 recordings at the neck connective (see Supplemental Experimental Procedures for an explanation

421 of the measurement of neural activity, a.u. = arbitrary units; mean + SD, N = 6 for each species,

422 \*\*\*:  $p < 0.001$ , student  $t$ -test).

423 (B) Threshold tuning curves for ascending neurons measured using intracellular recording  
424 methods (mean – SD; N=20 for *Gryllus* AN1 and AN2, N=11 for *Cardiodactylus* ANs). For  
425 comparison of thresholds, the black line represents thresholds for female vibrational responses to  
426 *Cardiodactylus* male call models. See Figure S2 for example recordings and Figure S3 for  
427 individual tuning curves.

428 (C) Axonal branches of ascending auditory neurons in the brain, reconstructed from intracellular  
429 stainings.

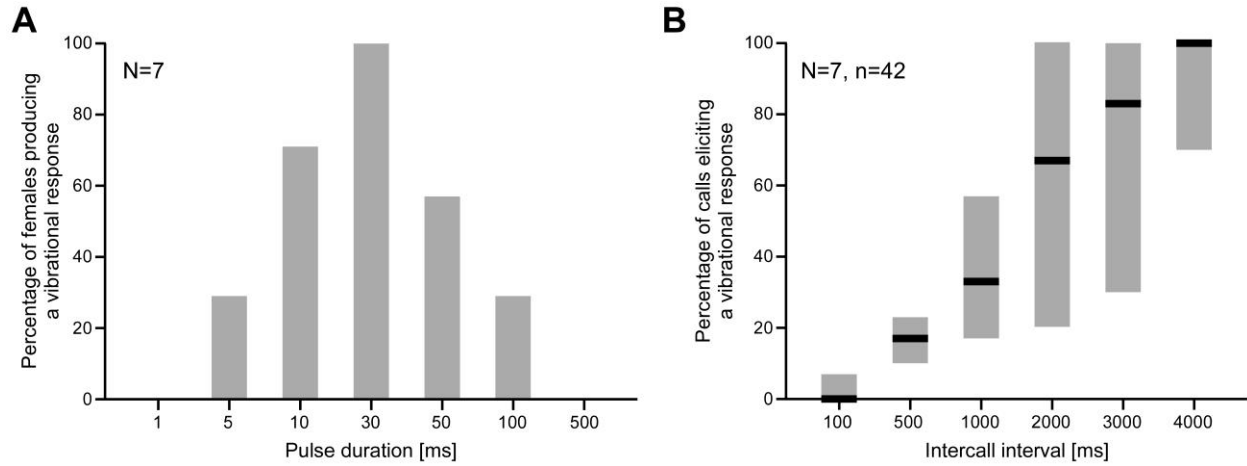


**Table 1. Behavioral responses to sound stimuli of females in eight cricket species.**

Latency values are means  $\pm$  SEM and different letters (superscript) indicate significantly different groups (Kruskal-Wallis test, Bonferroni correction for post hoc tests; *G. rubens* and *A. domesticus* were excluded from statistical analysis due to small sample sizes). See Figure S4 for a hypothesized sequence of events leading to the evolution of vibrational signaling in lebinthine crickets from an ASR precursor.

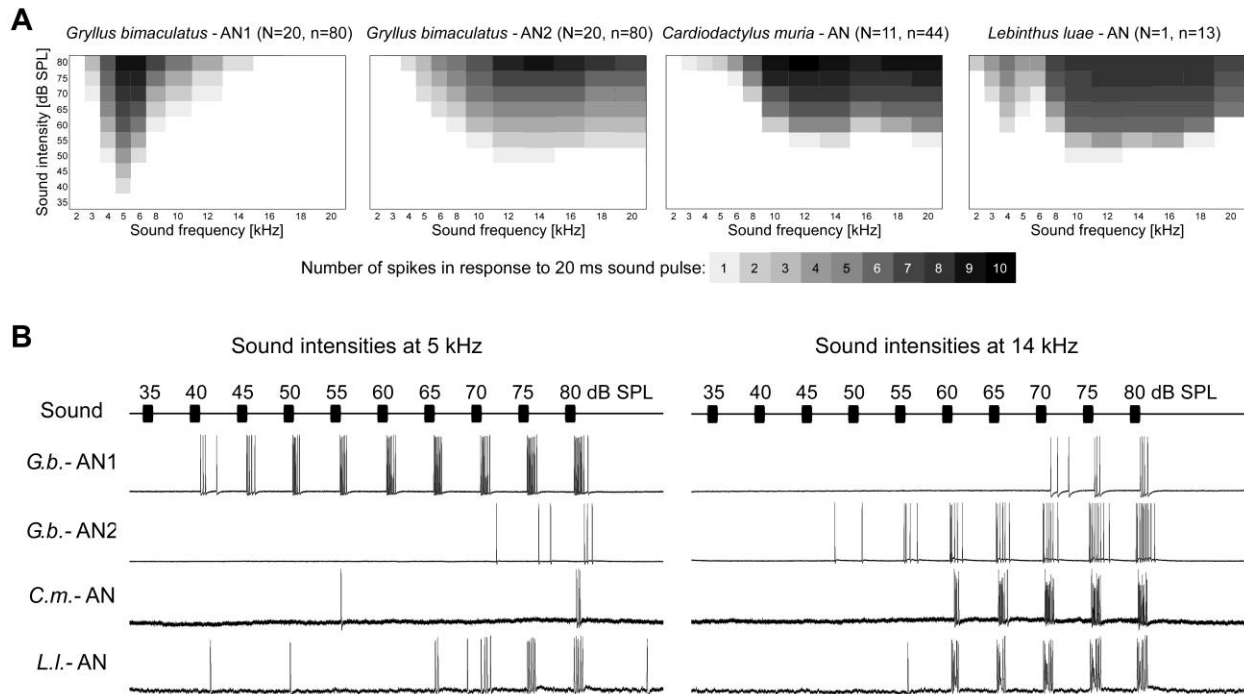
Subfamily	Species	Dominant frequency in male calling song [kHz]	Female behavior in response to male calling song	Percentage of individuals (N=6) showing ASR to 90 dB SPL sound pulse [%]	
				6 kHz	14 kHz
Gryllinae	<i>Gryllus bimaculatus</i>	5	Phonotaxis	0	100
	<i>Gryllus rubens</i>	5	Phonotaxis	0	33
	<i>Acheta domesticus</i>	5	Phonotaxis	0	50
	<i>Teleogryllus oceanicus</i>	5	Phonotaxis	0	100
Eneopterinae	<i>Nisitrus vittatus</i>	6	Phonotaxis	0	0
	<i>Cardiodactylus muria</i>	14	Vibrational reply	0	0
	<i>Agnotecous obscurus</i>	15	Vibrational reply	Not tested	Not tested
	<i>Lebinthus luae</i>	17	Vibrational reply	Not tested	Not tested

## SUPPLEMENTAL DATA



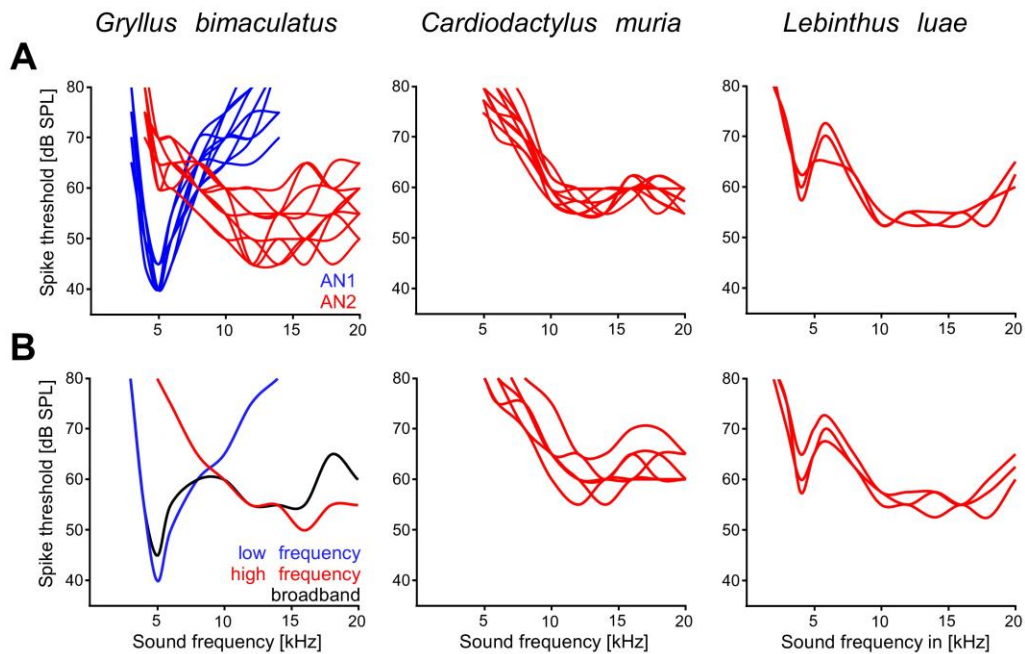
**Figure S1, related to Figure 2. Specificity of the *Cardiodactylus muria* female vibrational reply to call duration and call interval.**

All sound stimuli were 13 kHz sound pulses broadcast at 75 dB peak-equivalent SPL at the center of the cage. (A) Percentage of females producing a vibrational reply to sound pulses of varying durations repeated every 4 s (average male call duration:  $44.8 \pm 1.5$  ms,  $N=6$ ). (B) Percentage of sound pulses eliciting a female vibrational response for six 50 ms sound pulses repeated at different periods (average male calling song period:  $5.6 \pm 0.6$  s,  $N=6$ ). Black bars are median values; grey boxes are 10-90% percentiles ranges.



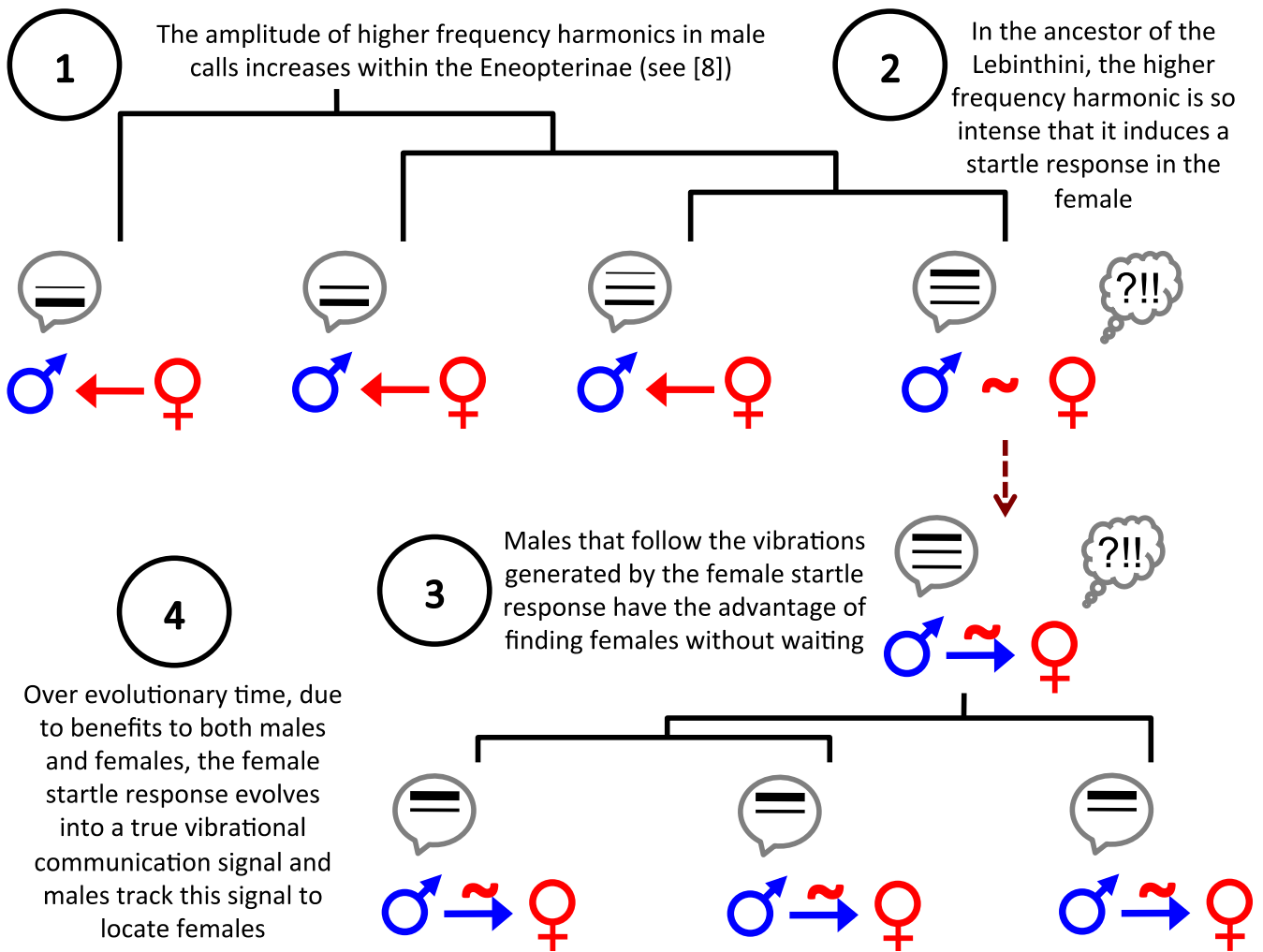
**Figure S2, related to Figure 3. Tuning curves and intracellular recordings of auditory ascending neurons in three cricket species.**

(A) Tuning curves showing the average number of action potentials produced in response to a 20 ms sound pulse at a range of frequencies and amplitudes (N = number of animals, n = number of sound presentations). (B) Examples of intracellular recordings of ascending neurons during playback of 5 kHz and 14 kHz sound pulses with increasing amplitude.



**Figure S3, related to Figure 3. Threshold tuning curves for auditory neurons based on intracellular recordings in the brains of three cricket species.**

(A) Ascending interneurons. (B) Brain neurons. For *Gryllus* and *Cardiodactylus*, each line is a recording from a different animal, for *Lebinthus* each line is a different recording from the same animal. Threshold was defined as an average response of at least one action potential. The decrease in threshold at ca. 4 kHz seen in *L. luae* is a feature that is also observed in recordings of AN2 neurons from several field cricket species (*Gryllus campestris* [S1], *Gryllus pennsylvanicus* [S2], *Teleogryllus commodus* [S3]).



**Figure S4, related to Table 1. Hypothesized evolutionary sequence of events leading from an acoustic startle response to a vibrational signal in lebinthine crickets.** Arrows indicate the direction of cricket movement, tildes indicates the production of substrate vibrations.

## 1 SUPPLEMENTAL EXPERIMENTAL PROCEDURES

2  
3 **Study animals:** Crickets were reared in the facilities of the Department of Zoology, University of Cambridge. Eneopterine colonies  
4 were derived from individuals or eggs collected in New Caledonia (*Agnotecous obscurus*), Singapore (*Lebinthus luae* and *Nisitrus*  
5 *vittatus*) and Indonesia (*Cardiodactylus muria*). The four field cricket species (*Acheta domesticus*, *Gryllus bimaculatus*, *Gryllus rubens*,  
6 *Teleogryllus oceanicus*) came from established captive breeding colonies.

7  
8 **Sound stimuli generation:** Sound stimuli were generated using Cool Edit Pro 2000 software (Syntrillium, Phoenix, USA, now Adobe  
9 Audition; Adobe Systems). Signals from a computer audio board were amplified with a custom-made amplifier and presented by  
10 speakers (Sinus Live NEO 13 S; Conrad Electronic, Wernberg-Köblitz, Germany). Peak equivalent sound pressure levels (peSPL: the  
11 r.m.s. level re.  $\mu$ 20 Pa of a sinusoid with the same peak-to-peak amplitude as the sound pulse [S4]) were calibrated for all sound stimuli  
12 at the position of the cricket to an accuracy of 1 dB (1/2" microphone type 4191 and measuring amplifier type 2610; Brüel and Kjær,  
13 Nærum, Denmark). Calling song of isolated males were recorded for each cricket species in a sound attenuating room using the same  
14 microphone and amplifier as above, a National Instruments AD board (PCI-MIO-16E4; NI, Austin, TX, USA) and Cool Edit software.  
15 The average values for pulse duration, pulse period (time from the start of one pulse to the start of the next), and call duration (time from  
16 the start of the first pulse to the end of the last pulse) were used as a guide to generate calling song models at single frequencies. The *C.*  
17 *muria* call model consisted of a single 50 ms pulse repeated every 5 s (Fig. 1). The *L. luae* call model had two parts separated by 380  
18 ms of silence: 1) a series of fifteen 20 ms pulses with a pulse period of 200 ms, and 2) a series of twenty-five 15 ms pulses with a pulse  
19 period of 25 ms. Total call duration was thus 3.815 s and calls were repeated every 15 s. The *A. obscurus* call model consisted of a single  
20 chirp containing fifty 16 ms pulses with a pulse period of 30 ms. Total call duration was thus 1.486 s and calls were repeated every 6.5  
21 s.



24 **Observations of female behavior in response to male calling song:** We observed cricket behavior under red light in a cylindrical  
25 fiberglass mesh arena (47 x 33 cm, L x D) placed in an anechoic chamber lined with sound attenuating foam. For phonotaxis trials, two  
26 speakers were placed in the anechoic chamber, one on each end of the cylindrical cage. Virgin female crickets were placed individually  
27 in the cage (*N. vittatus*, N=4; *C. muria*, N=8; *A. obscurus*, N=7; *L. luae*, N=9). A recording of species-specific male calling song was  
28 broadcast at 65 and 75 dB peSPL at the center of the arena for 20 minutes each from different ends of the cage, during which time the  
29 behavior of the cricket was observed. Behavior was scored as phonotaxis if the female walked within 5 cm of the end of the cage during  
30 playback of male calling song. Interactions between male and female lebinthine crickets were observed under red light in the same  
31 cylindrical arena described above (*C. muria*, N=13 pairs; *A. obscurus*, N=5 pairs). We waited for males to start singing or we stimulated  
32 male calling by broadcasting species-specific male song, and observed the couple for at least 20 minutes, or until the male and female  
33 found each other in the cage. During this time, we made detailed observations of the behavior of the male (calls, direction of walking  
34 behavior) and female (vibrational replies, walking behavior).

35  
36 **Measuring vibrational response tuning:** Unmated females of three eneopterine cricket species (*C. muria*, *A. obscurus*, *L. luae*) were  
37 placed in a vertically oriented cylindrical metal mesh cage (17 x 9 cm, L x D). Females of these species always perched on the vertical  
38 wall of the cage. An accelerometer (type 4393V, Brüel and Kjær, Nærum, Denmark) was attached to the side of the cage and the cage  
39 was placed in the same sound-attenuating chamber described above. Accelerometer signals were amplified (Nexus Conditioning  
40 Amplifier, Brüel & Kjær, Nærum, Denmark) and recorded using a National Instruments AD board (PCI-MIO-16E4; NI, Austin, TX,  
41 USA) and Labview software. Song models for each species were generated at 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 kHz. For each  
42 frequency, the call model was broadcast at 21 amplitudes (40-80 dB peSPL increasing in 2 dB steps). Each female was tested three times  
43 over three days with different orders for the frequencies: 1) increasing frequencies (2-20 kHz), 2) decreasing frequencies (20-2 kHz),  
44 and 3) staggered frequencies (2, 8, 14, 20, 4, 10, 16, 12 and 18 kHz). The order for these three trials was randomized for each cricket.  
45 The threshold for the female vibrational response to male calls was measured as the lowest sound level at which vibrations were



46 registered by the accelerometer. The threshold reported for each animal was the minimum threshold of these three trials. Response  
47 latency was measured as the time from the start of the last pulse in the male call to the start of the female vibrational signal. The frequency  
48 with the most energy in the vibrational reply was measured from power spectra generated in SASLabPro software (Avisoft Bioacoustics,  
49 Berlin, Germany).

50

51 **Measuring acoustic startle responses:** Female crickets were placed individually in a cylindrical metal mesh cage (17 x 9 cm, L x D).  
52 The cage was placed between a speaker and a 1/2" microphone and sound pulse amplitude was calibrated at the center of the cage. Pure-  
53 tone sound pulses of 100 ms duration (with 5 ms rising and falling ramps) and 90 dB SPL at the position of the cricket were presented  
54 at 6 or 14 kHz sound frequency. Sound stimuli were broadcast in random order for each cricket with 5 minutes of silence between  
55 stimuli. The beam of a Laser Doppler Vibrometer (PSV-400; Polytec, Waldbronn, Germany) was focused onto a piece of reflective foil  
56 on the cage, allowing for the detection of low amplitude vibrations in the cage generated by small movements of the cricket in response  
57 to sound. The presence or absence of vibrations within 500 ms following the stimulus was recorded, and the latency of the response was  
58 measured as the time from the start of the sound stimulus to the start of the vibration signal.

59

60 **Extracellular neural recordings:** Extracellular neural recordings were made with a double-hook electrode on the connective ipsilateral  
61 to the sound source and anterior to the prothoracic ganglion [S5]. The crickets were fixed ventral side up to a metal base and the front  
62 legs were waxed to wires holding them in a natural position [S6]. To reduce any neural activity unrelated to sound stimuli, both  
63 connectives posterior to the prothoracic ganglion and the contralateral connective anterior to the prothoracic ganglion were cut, and the  
64 ipsilateral connective was pinched with forceps anterior to the recording electrode. Sound stimuli were 50 ms pulses (with 5 ms rising  
65 and falling ramps) at 2, 3, 4, 5, 6, 8, 10, 12, 14, 16, 18, and 20 kHz with 200 ms silence between pulses, adjusted to 80 dB peSPL at the  
66 position of the cricket. The stimulation sequence was repeated 1000-2000 times for each cricket. Neural signals were amplified (DC  
67 amplifier BA-01X; NPI, Tamm, Germany) and digitally recorded with a National Instruments AD board (PCI-MIO-16E4; NI, Austin,

68 TX, USA) for storage and off-line analysis with Neurolab software [S7]. To extract the responses to sound from background neural  
69 activity, the absolute value of the voltage signal was used and averaged for each stimulus. The background activity was set to zero and  
70 the area under the neural response was calculated as a measure of overall neural activity in response to a sound pulse [S6]. To standardize  
71 the data across individual crickets, neural activity is presented as a relative response (area for the frequency divided by the mean area  
72 across all frequencies) (Fig. 3). We used t-tests to assess differences in neural activity between 5 and 14 kHz because these are  
73 frequencies that stimulate activity almost exclusively in AN1 and AN2, respectively.

74

75 **Intracellular recordings:** Intracellular recordings were performed in the brains of mature female crickets as previously described [S8].  
76 Sharp microelectrodes were pulled (DMZ-Universal Puller; Zeitz Instruments, Martinsried, Germany) from borosilicate glass capillaries  
77 (GC100F-10; Harvard Apparatus Ltd., Kent, UK). Neural activity was amplified (DC amplifier BA-01X; NPI, Tamm, Germany) and  
78 digitally recorded (A/D-converter Micro1401 mk II; CED, Cambridge, UK) for storage and off-line analysis with Spike2 software (CED,  
79 Cambridge, UK). For intracellular labeling, microelectrode tips were loaded with 5% Lucifer Yellow and dye was injected with 2-5 nA  
80 hyperpolarizing current [S9]. Brains were processed following standard histological protocols. Neurons were either photographed with  
81 a digital SLR camera (Canon EOS 350D) attached to a fluorescence microscope (Axioplan, Zeiss, Germany) or scanned with a confocal  
82 laser-scanning microscope (Leica SP5, Wetzlar, Germany). The morphology of neurons was graphically reconstructed from the digital  
83 image stacks using ImageJ 1.44 (National Institutes of Health, Bethesda, MD). Pure-tone sound pulses of 20 ms duration (with 1 ms  
84 rising and falling ramps) were presented at 2, 3, 4, 5, 6, 8, 10, 12, 14, 16, 18, 20 kHz sound frequency. For each frequency the sound  
85 intensity was systematically increased by steps of 5 dB from 35 to 80 dB SPL (80 ms silence between pulses).

86

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88 **SUPPLEMENTAL REFERENCES**

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