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Exploring the hidden landscape of female preferences for complex signals

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A major challenge in evolutionary biology is explaining the origins of complex phenotypic diversity. In animal communication, complex signals may evolve from simpler signals because novel signal elements exploit preexisting biases in receivers' sensory systems. Investigating the shape of female preference functions for novel signal characteristics is a powerful, but underutilized, method to describe the adaptive landscape potentially guiding complex signal evolution. We measured female preference functions for characteristics of acoustic appendages added to male calling songs in the grasshopper *Chorthippus biguttulus*, which naturally produces only simple songs. We discovered both hidden preferences for and biases against novel complex songs, and identified rules governing song attractiveness based on multiple characteristics of both the base song and appendage. The appendage's temporal position and duration were especially important: long appendages preceding the song often made songs less attractive, while following appendages were neutral or weakly attractive. Appendages had stronger effects on songs of shorter duration, but did not restore the attractiveness of very unattractive songs. We conclude that sensory biases favor, within predictable limits, the evolution of complex songs in grasshoppers. The function-valued approach is an important tool in determining the generality of these limits in other taxa and signaling modalities.

KEY WORDS: **Adaptive landscape, acoustic communication, complex signal, Orthopteran, sensory bias, signal evolution.**

The course of phenotypic evolution is directed by the adaptive landscape, that is, the function that relates trait values to fitness (Schluter 2000; Arnold et al. 2001). A major aim, and perennial challenge, in evolutionary biology is to determine how complex traits will respond to changes in the selective environment (Laughlin and Messier 2015). Studies of sexually selected signal evolution are particularly valuable toward this effort because the adaptive landscape for variation in male signal traits is in part determined by the shape of female preference functions, the function relating male signal characteristics to female response, which is readily estimated with standard mate preference experiments (Ritchie 1996; Wagner 1998). Inferences on the evolutionary effects of female preferences have been greatly facilitated by the development of new methods for the visualization and analysis of female preferences as function-valued traits (Stinchcombe and Kirkpatrick 2012). Function-valued approaches have been used to study the nature of sexual selection currently acting on male signal evolution in several species and signaling modalities (Blows et al. 2003; Rodríguez et al. 2006; Delcourt et al. 2010). In addition, the ease with which novel stimuli can be generated and presented (Basolo 1998; Burley and Symanski 1998) allows for studies of vast areas of previously uncharted space in the adaptive landscape surrounding the current distribution of male signal traits. Female preferences for novel signal phenotypes are important factors in the evolution of complex and multimodal signals (Rowe 1999; Candolin 2003; Hebets 2011), and measuring these preferences allows for the exploration of potential trajectories of future signal evolution (Arak and

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Enquist 1993, 1995). Nonetheless, little is known about the shape of female preference functions for novel signal elements. In this study, we characterize female preference functions for variation in the characteristics of novel acoustic appendages in male calling songs in the grasshopper *Chorthippus biguttulus biguttulus*.

Studies of sexual selection have focused particularly on the origin of female preferences (Kirkpatrick and Ryan 1991; Jennions and Petrie 1997; Kokko et al. 2003; Andersson and Simmons 2006; Prum 2012). Resolving this issue is important for predicting the effects of sexual selection on complex signals, defined as signals consisting of multiple distinct components (Rowe 1999), because the historical processes that led to the development of female preferences will influence the expression of preferences for novel signal variants (Ryan 1998; Phelps and Ryan 2000; Ryan et al. 2001). The sensory bias hypothesis proposes that female preferences for complex signals arose as incidental by-products of receiver sensory processing mechanisms (Ryan 1990; Endler and Basolo 1998; Ryan and Cummings 2013). Receivers may have "hidden" preferences for certain novel signals because the algorithms used to evaluate mating signals lead to biases toward exaggerated or supernormal versions of the signal characteristics under selection (Arak and Enquist 1993; Enquist and Arak 1993; Phelps and Ryan 1998), and because sensory systems are often biased toward signals that mimic the characteristics of stimuli that are important in other contexts such as foraging or predator avoidance (Ryan 1990; Endler and Basolo 1998). Hidden preferences have important implications for evolutionary biology because the potential for complex signal evolution can be explored in species that do not currently produce such signals, giving clues into the origins of these complex traits (Witte et al. 2001; Gerhardt et al. 2007a,b; Gerhardt and Humfeld 2013).

Sensory biases are an important force in the evolution of signal diversity (Ryan 1998; Fuller et al. 2005), but because these biases often favor signal characteristics that are not normally expressed, it is challenging to predict which types of traits could be favored, or the resulting strength and direction of selection. In a survey of female preferences for male acoustic and visual signal traits, when directional preferences for signal complexity were present, they always favored more complex signals (Ryan and Keddy-Hector 1992). Thus, one version of the sensory bias hypothesis proposes that any signal elaboration that results in greater stimulation of the female's sensory system will be favored by sexual selection (Ryan and Rand 1990; Rowe 1999). Females are predicted to be more responsive to signals with novel elements, but discriminate little between different characteristics of these signals, resulting in a relatively elevated, but flat preference function. However, mechanisms of sensory processing are not necessarily biased toward complexity per se, and instead may involve more sophisticated rules that govern the attractiveness of signal elaborations, thus limiting the possible trajectories of complex signal evolution (Guilford and Dawkins 1991; Miller and Bee 2012). In this scenario, the female preference function will be sloped, because only a limited range of novel signal elements render complex signals more attractive; outside of this range the resulting complex signals may even be unattractive. Few studies have attempted to test the predictions of these different variations of the sensory bias hypothesis (Gerhardt et al. 2007a).

For long-range mate attraction, male *C. b. biguttulus* produce a simple calling song by stridulation, generating a repeated series of syllables and pauses (von Helversen and von Helversen 1997). When females find a male song attractive they produce a response song, which allows the male to approach the female and initiate close-range courtship (von Helversen 1997). Female response singing makes *C. biguttulus* ideal for exploring the adaptive landscape for novel signals because the attractiveness of a song can be measured automatically and for dozens of stimulus presentations within an experimental session (Schmidt et al. 2008). Furthermore, females respond readily to synthetic songs, which enables experimental manipulation of individual signal characteristics while others are held constant (von Helversen 1972; von Helversen and von Helversen 1997). Although male *C. b. biguttulus* do not produce complex songs (i.e., their songs do not contain multiple acoustic subunits; Fig. 1A), other closely related grasshoppers do (Vedenina and Mugue 2011). In particular, *C. biguttulus hedickei*, a subspecies of *C. biguttulus*, produces a complex song in which the normal calling song is often followed by an appendage with different acoustic characteristics (Fig. 1B; von Helversen 1989). Female *C. b. biguttulus* are highly selective for multiple temporal features of male songs (von Helversen and von Helversen 1994; Balakrishnan et al. 2001; Klappert and Reinhold 2003), and this selectivity varies across the duration of the song: unattractive syllables at the song's beginning have a stronger negative influence on female response than the same unattractive syllables at the song's end (Clemens et al. 2014). Thus, although male *C. b. biguttulus* produce only simple songs, there is reason to expect hidden female preferences for complex songs, but also to expect that such preferences will favor only a limited range of temporal characteristics of novel song elements.

We measured the shape of female preference functions for synthetic complex songs that consisted of a novel acoustic appendage added to a standard (simple) male calling song model. The appendage could either precede or follow the standard song. Our primary aim was to test whether females have sensory biases for novel complex songs, and if so to discriminate between sensory biases favoring complexity per se and those in which there are rules governing complex song attractiveness based on temporal characteristics of the novel appendage. To test this, we generated preference functions for complex songs with appendages

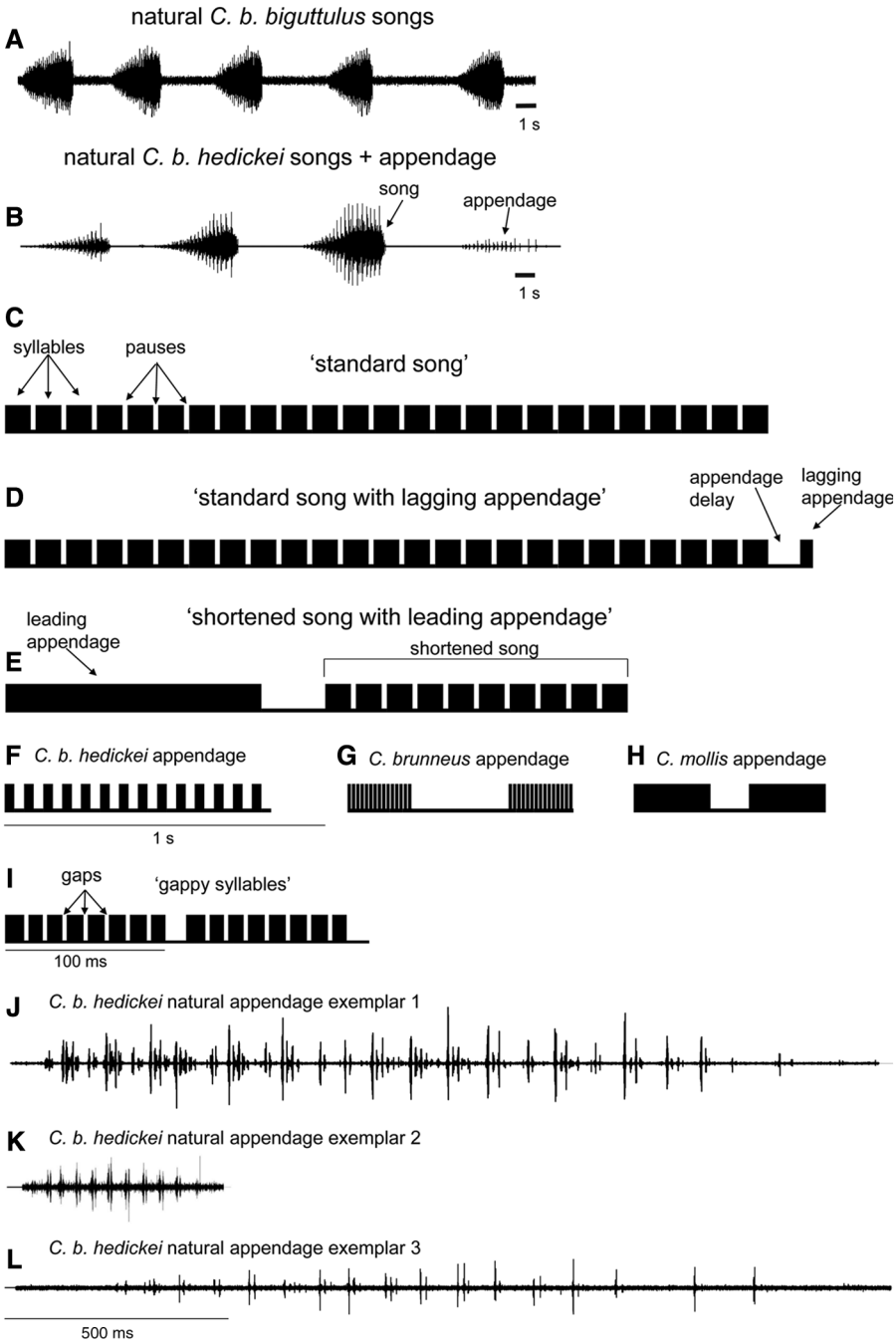

Figure 1. **Waveforms illustrating the characteristics of some of the playback stimuli. Each graph shows the pattern of amplitude (*y*-axis, relative amplitude, linear units) over time (*x*-axis). (A) A recording of five natural male *C. b. biguttulus* songs. (B) A recording of three natural male *C. b. hedickei* songs plus an acoustic appendage to the calling song. Both (A) and (B) were recorded at approximately 30°C (C). The standard song stimulus, illustrating the syllable-pause structure of typical male *C. b. biguttulus* song. A song consists of a repeated series of syllables, separated by pauses (indicated with arrows). (D) The standard song with a lagging noise appendage. In this particular example, the appendage duration is 40 ms and the appendage delay is 100 ms. (E) The shortened song with a leading noise appendage. In this particular example, the appendage duration is 800 ms and the appendage delay is 200 ms. (F) The synthetic *C. b. hedickei* appendage. (G) The synthetic *C. brunneus* appendage. (H) The synthetic *C. mollis* appendage. (I) Close-up of two syllables of the gappy song stimulus. The complete stimulus contained 25 such syllables. Each syllable is interrupted by seven silent gaps. Stimuli (C)–(I) are illustrated schematically to have no amplitude modulation whatsoever at the syllable peak, but the actual stimuli used in the experiments would have contained minor random fluctuations in amplitude across the syllable peak because of the random noise generation process. (J–L) The three natural exemplars of *C. b. hedickei* appendages. Note the different time scales for (A–B), (C–H), (I), and (J–L).**

varying in multiple temporal characteristics and assessed whether the function was flat (complexity per se hypothesis) or sloped, favoring only certain temporal characteristics (attractiveness rules hypothesis). We performed additional experiments to further examine the potential for complex signal evolution in this species. First, we tested female preferences for appendages that resemble signal elements produced by a closely related subspecies (*C. b. hedickei*, which naturally produces complex songs with acoustic appendages; Fig. 1B) and two heterospecifics (*C. brunneus* and *C. mollis*). Second, we tested whether appendages "rescue" the attractiveness of calling songs (see Seeba et al. 2010; Henderson and Gerhardt 2013) that were otherwise less attractive than the standard because they were shorter in duration (Ronacher and Stange 2013), or that were highly unattractive because the temporal pattern of syllables was interrupted by silent gaps (von Helversen 1972). In sum, we present an extensive exploration of hidden preference space, uncovering important insights into the rules governing the evolution of signal diversity.

Methods

We tested unmated female *C. b. biguttulus* ($N = 151$) that were collected as juveniles in the field during the natural breeding season (July—September 2014, 2015) from populations near Göttingen (N 51°28′10.41″, E 9°56′24.98″) and Berlin (N 52°32′3.33″, E 13°40′23.01″), Germany, and Kühtai, Austria (N 47°12′57.5″, E 11°01′50.9″). We reared the females to sexual maturity in group houses with no males. We also tested females raised in the laboratory from eggs collected near Göttingen in 2013 and 2014. Females were marked with combinations of painted dots on different body parts to ensure individual identification. Females were maintained between 22—28˚C and fed fresh grass and fish food flakes ad libitum.

STIMULUS PREPARATION

We generated synthetic songs using a custom program in LabView 7.0 software (National Instruments, Texas; programmed by R. Matthias Hennig). The standard song model mimicked a natural, moderately attractive male *C. b. biguttulus* calling song (von Helversen and von Helversen 1997), and consisted of a repeated series of syllables and silent pauses (syllable duration: 80 ms, syllable rise and fall time: 1 ms, pause duration: 16 ms). The entire song contained 25 syllables for a total duration of 2.4 s (Fig. 1C). Song stimuli were synthesized by generating the amplitude envelope of the calling song and filling it with broadband noise filtered to contain frequencies between 4.5 and 40 kHz. The use of a synthetic song model allowed us to precisely control song characteristics to approximate an average male song, and to avoid the issue of pseudoreplication associated with statistical analyses of responses to natural song variants (McGregor 1992).

To generate the full set of experimental stimuli (complete description in Table S1), we modified one or more of the following characteristics of the standard song: 1. Song duration: The standard song contained 25 syllable-pause repetitions; the "shortened" song had only 10 syllable-pause repetitions (Fig. 1C, E). 2. Syllable gaps: Males that lose a hindleg produce songs with discontinuous syllables containing short gaps between sound pulses (Elsner 1974); females discriminate strongly against such songs (von Helversen and von Helversen 1997). The standard song had no gaps within syllables, while each syllable of the "gappy" song was interrupted by 7 evenly-spaced silent gaps of 3 ms (Fig. 1I). The total acoustic energy of the syllables was equalized for both stimuli, thus, each gappy song syllable was 21 ms longer than the standard song syllables. 3. Appendage position. Appendages were either placed before the calling song ("leading" appendages; Fig. 1E) or after the calling song ("lagging" appendages; Fig. 1D). 4. Appendage delay. We varied the amount of time elapsing between the appendage and the calling song (Table S1). The delay was measured as the time between the appendage's offset and the song's onset for leading appendages and the song's offset and the appendage's onset for lagging appendages. 5. Appendage type. Most trials were conducted with "noise" appendages, consisting of a single, unmodulated syllable with the same amplitude and frequency characteristics as the syllables of the standard song. We used noise appendages because calling songs consisted of similar signal elements. We tested the effects of appendage duration by synthesizing noise appendages at 12 different durations (Table S1). We also conducted trials with synthetic "heterospecific" appendages, which were modeled after portions of the songs of *C. brunneus* and *C. mollis*, and after the appendage naturally produced by *C. b. hedickei*. The *C. b. hedickei*-like appendage consisted of 14 syllables of 30 ms separated by 30 ms pauses (total duration: 810 ms; Fig. 1F). The *C. brunneus* appendage consisted of two phrases of male calling song, each containing 15 repetitions of 10 ms syllables with 3 ms pauses. The phrases were separated by 300 ms (total duration: 690 ms; Fig. 1G). The *C. mollis* appendage consisted of two syllables of 240 ms separated by a 120 ms pause (total duration: 600 ms; Fig. 1H). Finally, we conducted some trials with three different natural exemplars of *C. b. hedickei* appendages (Fig. 1J–L) obtained from field recordings (recording details in Table S2).

TEST PROCEDURE

Playbacks were controlled by a custom software program that automatically broadcast male songs and recorded female responses (full details of the playback setup in Schmidt et al. 2008). All tests were performed at 30˚C. Females were placed in a small acoustically transparent cage within a chamber lined with

echo-reducing foam. Calling songs were broadcast through a dome tweeter loudspeaker positioned laterally to the female's cage. The sound-pressure level (SPL) was calibrated in the center of the female's cage with a Brüel and Kjær (Nærum, Denmark) sound level meter (Type 2231) and condenser microphone (Type 4133). Female response songs were recorded onto the computer via a tie-clip microphone suspended approximately 3 cm above the female's cage. Females reliably signal their attraction to male calling songs by producing response songs (von Helversen and von Helversen 1994; Klappert and Reinhold 2003). We considered a female to have responded to a stimulus presentation if it sang at least once in the 25 s following stimulus playback.

During an experimental session, females were exposed to 18–20 repetitions of the set of experimental stimuli; the specific stimuli used depended on the experiment and are detailed below. All of the stimuli in a given experiment were broadcast once, in random order, before being rerandomized on each subsequent repetition. The combination of stimulus randomization and the large number of stimulus repetitions controlled for any order effects. Prior to each repetition, female motivation was tested by presenting an attractive control stimulus; if females failed to respond to this stimulus, testing was paused for 2 minutes before the next presentation of the control stimulus. We quantified each female's response to each stimulus as the proportion of presentations to which she gave a response song out of the total number of presentations of that stimulus. A female's response measurements were only included in analyses if it was sufficiently motivated; in line with previous studies we defined this as a response to at least half of the presentations of at least one experimental stimulus (Schmidt et al. 2008; Reichert and Ronacher 2015).

Our hypotheses for the shape of female preference functions were based on comparisons between the baseline response to the standard song and responses to novel complex songs; we were primarily interested in changes in preference function shape resulting from additions of acoustic appendages. We therefore quantified the relative attractiveness of complex songs by subtracting each female's response to each complex stimulus from its response to the corresponding stimulus without an appendage, and using this difference as the dependent variable in our statistical models and to generate preference functions. Analyses using absolute response measures obtained qualitatively similar results to those presented here (Table S3).

EXPERIMENT 1 – STANDARD SONGS WITH NOISE APPENDAGES

We added noise appendages to the standard calling song to test the hypotheses that the attractiveness of novel complex songs depends on appendage duration, appendage position, and appendage delay. In a single experimental session we measured female responses to complex songs with two of the 12 appendage durations (Table S1) at all combinations of appendage position (two levels) and appendage delay (seven levels). As controls, we also measured each female's response to the standard song with no appendage, each of the two tested appendages with no accompanying song, 3.24 s of unmodulated noise, and a silent stimulus. The latter two stimuli were negative controls to ensure that females were not responding indiscriminately: females normally do not respond to unmodulated noise and rarely sing spontaneously (von Helversen 1972). These 33 stimuli were presented in random order; successive presentations were rerandomized until the female had been tested for a total of 20 repetitions of each stimulus. The intensity of all stimuli was adjusted so that the syllable plateau was broadcast at 70 dB (peak SPL), an SPL at which females are highly responsive (von Helversen and von Helversen 1997; Balakrishnan et al. 2001). Our data include three trials where a female responded to only 15, 18, and 19 complete stimulus repetition cycles, respectively, because an error in the computer program terminated the playback cycle early. Females were sometimes tested again either on the same day or a different day at additional appendage durations; as above, their motivation was probed throughout testing to ensure they remained responsive to acoustic signals.

EXPERIMENT 2 – SHORTENED SONGS WITH NOISE APPENDAGES

We tested whether appendage duration and position affects the attractiveness of shortened *C. b. biguttulus* songs. We measured each female's response to shortened songs with appendages of five different durations in the leading or lagging position (Table S1). Appendage delay was held constant at 200 ms for all stimuli. We also measured each female's response to the standard song with no appendage, the shortened song with no appendage, four additional attractive songs, and unmodulated noise. The intensity of all stimuli was adjusted so that the syllable plateau was broadcast at 64 dB (peak SPL). The 17 stimuli for this experiment were broadcast along with nine stimuli from Experiment 4. Stimuli were randomized as in Experiment 1 and all females were given 18 repetitions of each stimulus. The response variable was the difference between each female's response to the complex stimulus and its response to the shortened song with no appendage.

EXPERIMENT 3 – GAPPY SONGS WITH NOISE APPENDAGES

We tested whether appendage duration, appendage delay, or appendage position restored the attractiveness of the normally unattractive gappy song. In each experimental session, we measured each female's response to complex songs in which noise appendages were added to the gappy song with two of eight appendage durations (Table S1) at all combinations of appendage position (two levels) and appendage delay (seven levels). We also measured each female's response to the standard song with no

appendage, each of the two appendages with no accompanying song, the unmodulated noise and silent stimuli from Experiment 1 and four attractive song stimuli. The attractive song stimuli helped maintain female motivation, which may otherwise have waned because of the high proportion of stimulus presentations with unattractive gappy songs. These 37 stimuli were presented at 70 dB and randomized as in Experiment 1. Our data include two trials where a female was presented with only either 14 or 19 complete stimulus repetition cycles. If appendages rescue the attractiveness of gappy songs, then females should respond similarly to complex gappy songs and the standard song. Thus, the response variable for analyses was the difference between the female's response to each stimulus and its response to the standard song.

EXPERIMENT 4 – HETEROSPECIFIC APPENDAGES

We tested whether appendages resembling portions of natural signals produced by *C. b. hedickei* and two closely related heterospecifics (for simplicity, together referred to as "heterospecific" stimuli) influence the attractiveness of calling songs in *C. b. biguttulus*. We manipulated appendage type (six levels, see below), song duration (standard or shortened), and appendage position (leading or lagging). Appendage delay was held constant at 200 ms. One set of females was tested with the shortened song with a synthetic appendage of either *C. b. hedickei*, *C. brunneus* or *C. mollis* in the leading or lagging position. These females were also tested with the three synthetic heterospecific appendages without an accompanying song to determine if the appendage stimuli alone were attractive. These nine stimuli were presented along with 17 stimuli from Experiment 2, and randomized as in Experiment 1. A second set of females was tested with the standard song with each of the three synthetic heterospecific appendages in the leading or lagging position, and both the standard and shortened song with each of the three natural *C. b. hedickei* appendages in the leading or lagging position. We also measured responses to the standard song, the six heterospecific appendages without an accompanying song, unmodulated noise, and four additional attractive songs. An error in the computer program only detected after completing the experiments resulted in faulty playback of the stimulus with a synthetic lagging *C. b. hedickei* appendage added to the standard song. Female responses to this stimulus were therefore not analyzed. Stimuli were broadcast at 64 dB peak SPL.

We tested the hypothesis that the influence of heterospecific appendages on song attractiveness depends on song duration and appendage position. We performed separate analyses for natural and synthetic heterospecific stimuli. For natural appendages, we included appendage exemplar as an effect to assess whether females responded differently to the three exemplar recordings. For synthetic appendages, we included species as an effect to determine if females responded differently to synthetic appendages containing song elements of each species. The response variable was the difference between the female's response to the complex stimulus and its response to the song of corresponding duration without an appendage.

DATA ANALYSIS

For Experiments 1 and 3 we analyzed the shape of female preference functions for temporal appendage characteristics. We used the lmer function in the lme4 package (version 1.1–10; Bates et al. 2015) in R software (version 3.2.2; R Development Core Team 2015) to perform linear-mixed models fitted with maximum likelihood with the appendage characteristics (duration, delay, position) as fixed effects. We included quadratic terms for appendage duration and delay to estimate nonlinear selection, along with all possible two-way interactions between effects. Female identity was included as a random effect in all models to account for repeated measurements. The response variable was the difference between each female's response to the given complex stimulus and its response to the standard song. The statistical significance ($\alpha = 0.05$) of individual effects was evaluated with the *t*-statistic output from the model obtained with the lmerTest package (version 2.0–32; Kuznetsova et al. 2016). When higher level interaction terms were not statistically significant, they were removed from the model. We visualized preference functions graphically with cubic splines generated from general additive models (generalized cross-validation method) using the mgcv package (version 1.8–7; Wood 2011) in R. Separate splines were plotted for leading and lagging appendages. For Experiments 2 and 4, we carried out linear-mixed models, as above, but did not include quadratic terms or plot splines because Experiment 2 had only 10 stimulus variants, and because the stimuli of Experiment 4 differed qualitatively. For these experiments, we present the response to each stimulus graphically as the average response across all females bounded by 95% confidence intervals (for similar presentations for Experiment 1 and 3, see Figs. S1–S15).

Preliminary analyses indicated differences in female response depending on whether the appendage was in the leading or lagging position. To further illustrate this finding, we calculated the mean attractiveness ($\pm$ 95% CI) of each stimulus (the mean of the differences between each female's response to a given complex song and the simple song). We combined results from Experiments 1, 2, and 4, and for each stimulus categorized the appendage's effect as positive, neutral, or negative if the confidence interval of the difference between the response to the complex song and the simple song was positive and did not cross zero, crossed zero, or was negative and did not cross zero, respectively. We tabulated the number of leading- and lagging-appendage stimuli in each of these categories.

Raw data from this study are available from the Dryad data repository (doi: 10.5061/dryad.51g4t/1).

Table 1. Results of linear-mixed models testing effects of appendage and song characteristics on the difference between the proportion of responses to complex songs and the proportion of responses to the corresponding song with no appendage.

Experiment	Fixed effect	Parameter estimate (±SE)	df	*t*	*P*
1. Noise appendage, standard song	Intercept	0.110 ± 0.021	100	5.4	**<0.001**
	Duration	$-3.56 \times 10^{-4} \pm 5.30 \times 10^{-5}$	3139	–6.7	**<0.001**
	Delay	$-1.52 \times 10^{-4} \pm 9.84 \times 10^{-5}$	3100	–1.5	0.12
	Position	0.012 ± 0.012	3100	1.0	0.32
	Duration^2	$-3.55 \times 10^{-7} \pm 6.72 \times 10^{-8}$	3138	5.3	**<0.001**
	Delay^2	$4.67 \times 10^{-8} \pm 2.35 \times 10^{-7}$	3100	0.2	0.84
	Duration × delay	$1.71 \times 10^{-7} \pm 6.41 \times 10^{-8}$	3100	2.67	**0.008**
	Duration × position	$-5.55 \times 10^{-4} \pm 6.56 \times 10^{-5}$	3100	–8.46	**<0.001**
	Delay × position	$-3.37 \times 10^{-4} \pm 1.37 \times 10^{-4}$	3100	–2.47	**0.014**
	Duration^2 × Position	$3.51 \times 10^{-7} \pm 8.49 \times 10^{-8}$	3100	4.13	**<0.001**
	Delay^2 × Position	$6.58 \times 10^{-7} \pm 3.33 \times 10^{-7}$	3100	1.98	**0.048**
2. Noise appendage, shortened song	Intercept	0.074 ± 0.023	52	3.2	**0.003**
	Duration	$1.03 \times 10^{-5} \pm 3.29 \times 10^{-5}$	261	0.3	0.76
	Position	-0.093 ± 0.019	261	–4.8	**<0.001**
	Duration × position	$-1.97 \times 10^{-4} \pm 4.65 \times 10^{-5}$	261	–4.2	**<0.001**
3. Noise appendage, gappy song	Intercept	-0.47 ± 0.040	49	–11.9	**<0.001**
	Duration	$-2.43 \times 10^{-4} \pm 3.61 \times 10^{-5}$	1864	–6.7	**<0.001**
	Delay	$-3.12 \times 10^{-5} \pm 2.28 \times 10^{-5}$	1830	–1.4	0.17
	Position	-0.033 ± 0.0062	1830	–5.2	**<0.001**
4. Synthetic heterospecific appendage	Intercept	-0.056 ± 0.025	100	–2.2	**0.03**
	Species (*C. b. hedickei*)	0.052 ± 0.017	281	3	**0.003**
	Species (*C. mollis*)	0.011 ± 0.016	281	0.7	0.5
	Position	-0.21 ± 0.014	281	–15.2	**<0.001**
	Song duration	0.11 ± 0.033	64	3.3	**0.001**
4. Natural *C. b. hedickei* appendage	Intercept	0.084 ± 0.034	170	2.4	**0.015**
	Exemplar 2	-0.084 ± 0.037	374	–2.2	**0.026**
	Exemplar 3	-0.10 ± 0.037	374	–2.8	**0.005**
	Position	-0.073 ± 0.035	374	–2.1	**0.038**
	Song duration	-0.23 ± 0.035	374	6.5	**<0.001**
	Position × song duration	0.093 ± 0.035	374	2.6	**0.009**
	Position × exemplar 2	-0.013 ± 0.043	374	–0.3	0.75
	Position × exemplar 3	0.0090 ± 0.043	374	0.2	0.84
	Song duration × exemplar 2	-0.043 ± 0.043	374	–1	0.32
	Song duration × exemplar 3	-0.050 ± 0.043	374	–1.2	0.25

Only the final models after the removal of nonsignificant higher order interaction terms (see text for details) are shown here. Fixed effects refer to characteristics of appendages, except for song duration, which refers to the duration of the song itself and was modeled as a categorical fixed effect with two levels (standard and shortened song, standard is the reference category). Position is modeled as a categorical fixed effect with two levels (lagging and leading appendage position, lagging is the reference category). Appendage duration and delay were measured in milliseconds prior to centering and scaling (see Methods). Significant *P* values are highlighted in bold.

Results

EXPERIMENT 1 – STANDARD SONGS WITH NOISE APPENDAGES

We conducted a total of 113 sessions in this experiment, using 63 different females. Females in this experiment responded to the standard song with no appendage on average (± SD) to 52.3 ± 20.1% of the presentations. The attractiveness of the noise-appendage stimuli was affected by two-way interactions between appendage position and both the linear and quadratic effects of appendage duration and appendage delay (Table 1). The preference function plots clearly illustrate the strong interaction effects of appendage duration and position on song attractiveness (Fig. 2). Leading appendages were almost always either neutral or had negative effects on song attractiveness. Longer duration leading appendages, except for those with a duration of 500 and 600 ms, were especially unattractive. The interaction effect between appendage duration and position was negative, indicating that the disparity in female responsiveness to a leading

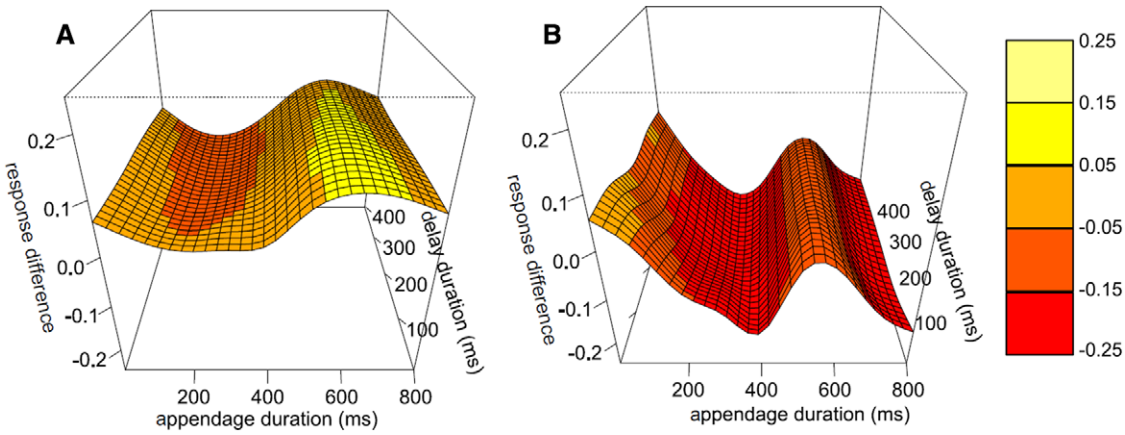

Figure 2. **Preference functions illustrating variation in female response to novel complex songs in which we varied the duration, delay, and position of noise appendages added to the standard song stimulus. Appendages were placed either (A) after the song (lagging appendages) or (B) before the song (leading appendages). Each chart shows the spline function for variation in female response (difference between response to complex stimulus and response to standard song, which had no appendage) as predicted from a general additive model with appendage duration and delay duration as factors. The relative attractiveness of complex stimuli is depicted both as the elevation along the *z*-axis (positive values indicate that females responded more often to the complex song than to the standard song; negative values indicate that females responded more often to the standard song than to the complex song) and as the color of individual grid cells; darker cells indicate a less attractive stimulus (see legend). Sample sizes are given in Table S1. Raw averages and 95% confidence intervals of female responses to each combination of appendage position, delay, and duration for these stimuli are shown in Figures S1–S7.**

appendage compared to the same lagging appendage increased with appendage duration. The interaction effect between appendage duration^2 and position was positive, indicating a stronger curvilinear relationship between these two variables for leading appendages. Appendage duration had moderate effects on song attractiveness for lagging appendages, while the effects of appendage duration on song attractiveness were strong and nonlinear for leading appendages (Fig. 2). Although there were statistically significant interaction effects between appendage position and both linear and quadratic appendage delay, and between appendage position and delay, these were relatively minor compared to the effects of appendage duration (Fig. 2). The most prominent effect of appendage delay was that leading, short-duration appendages at shorter delays had neutral or occasionally positive effects on song attractiveness, while leading, short-duration appendages at longer delays were generally neutral or less attractive than the standard song (Figs. 2, S1–S7). None of the noise appendage stimuli were attractive when presented alone (Fig. S8).

EXPERIMENT 2 – SHORTENED SONGS WITH NOISE APPENDAGES

We measured the responses of 29 different females to shortened songs with noise appendages. Females responded significantly more often to the standard song (mean ± SD response, 65 ± 24%) than to the shortened song (32 ± 25% response; mean ± SD difference in response, 33.2 ± 24.2%; paired *t*-test, $t_{28} = 7.4$, $P < 0.001$). There was a significant interaction effect between appendage duration and position on the attractiveness of shortened-song stimuli (Table 1). Each of the lagging appendages had a similarly positive effect on song attractiveness (Fig. 3). For leading appendages, however, short-duration appendages were neutral and appendages 200 ms or longer had negative effects on song attractiveness (Fig. 3).

EXPERIMENT 3 – GAPPY SONGS WITH NOISE APPENDAGES

We conducted a total of 67 sessions in this experiment, using 46 different females. No combination of appendage characteristics restored the attractiveness of a gappy song to anywhere near the level of attractiveness of the standard song (Fig. 4, S9–S15). The attractiveness of the gappy songs with noise appendages was not significantly affected by any two-way interactions or quadratic effects of appendage characteristics so these terms were removed from the model. In the final model, there were statistically significant effects of appendage duration and position, but not appendage delay, on the attractiveness of gappy songs (Table 1). Nonetheless, the magnitude of these effects on responses to gappy songs was rather small compared to the large effect of gaps in rendering a song unattractive.

EXPERIMENT 4 – HETEROSPECIFIC APPENDAGES

We measured the response of 29 females to shortened songs with synthetic heterospecific appendages and 34 females to the standard song with synthetic heterospecific appendages. The

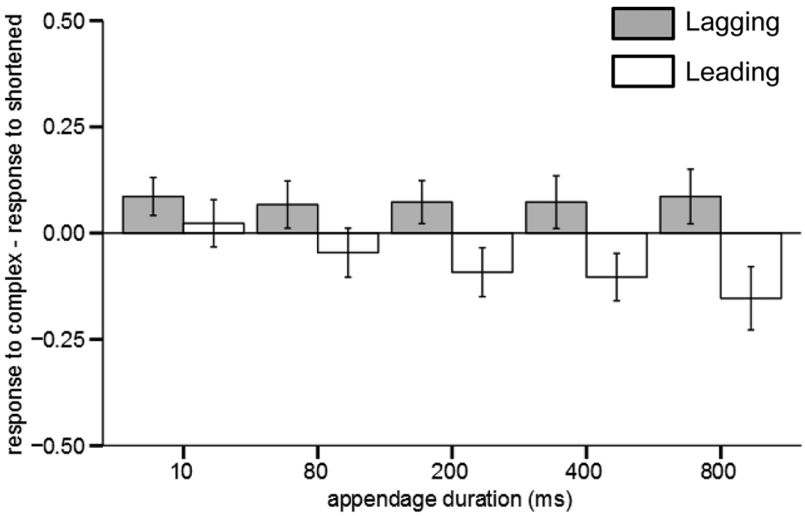

Figure 3. Responses of females to complex songs with noise appendages of varying duration, appended to the shortened song stimulus. Lagging appendages are depicted with gray bars, leading appendages are depicted with white bars. The appendage delay was 200 ms. Each bar shows the mean (± 95% confidence interval) of the differences between each female's response to the given complex stimulus and its response to the shortened song, which had no appendage. $n = 29$ females.

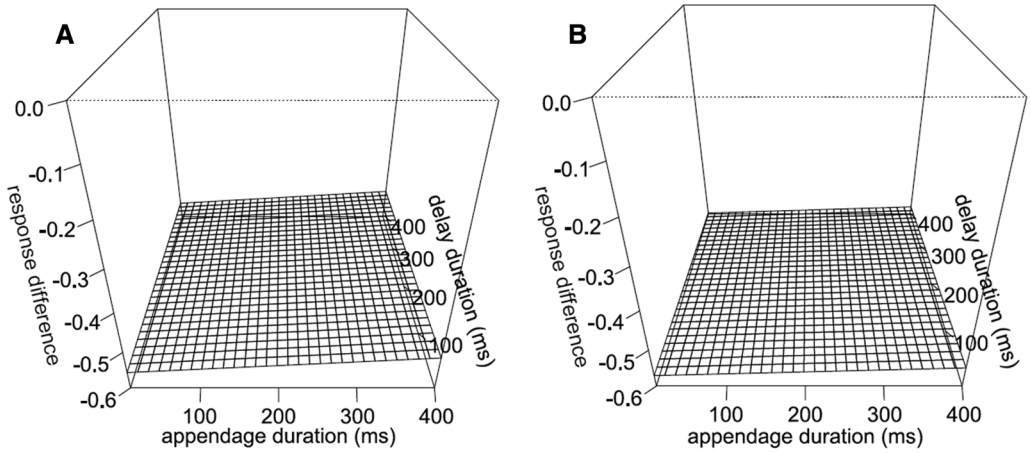

Figure 4. Preference functions illustrating variation in female response to novel complex songs in which we varied the duration, delay, and position of noise appendages added to the gappy song stimulus. (A) Lagging appendages, (B) Leading appendages. Each chart shows the spline function for variation in female response as in Figure 2. We do not use colors to illustrate the relative attractiveness of the complex stimuli because there was little variation in female response to these stimuli. Sample sizes are given in Table S1. Raw averages and 95% confidence intervals of female responses to each combination of appendage position, delay, and duration for these stimuli are shown in Figures S9–S15.

attractiveness of these stimuli was not significantly affected by the interaction between song duration, appendage position, and appendage species, or by any two-way interactions so these terms were removed from the model. In the final model, there were statistically significant effects of appendage position, song duration, and species (Table 1). For the standard song, leading synthetic heterospecific appendages were always strongly unattractive, while lagging appendages were weakly unattractive (Fig. 5A). Leading synthetic heterospecific appendages were also strongly unattractive when added to the shortened song (Fig. 5B). However, lagging synthetic heterospecific appendages moderately improved the attractiveness of the shortened song (Fig. 5B). Females responded slightly more to the *C. b. hedickei* stimulus than to the *C. brunneus* stimulus (Table 1, Fig. 5B).

We obtained responses of 34 females to songs with the three natural *C. b. hedickei* appendage exemplars. The attractiveness of these complex songs was not significantly affected by the interaction between song duration, appendage position, and exemplar, so this term was removed from the model. In the subsequent model, there was a statistically significant interaction effect of appendage position and song duration (Table 1). For the standard song, two of the leading *C. b. hedickei* exemplars made the song

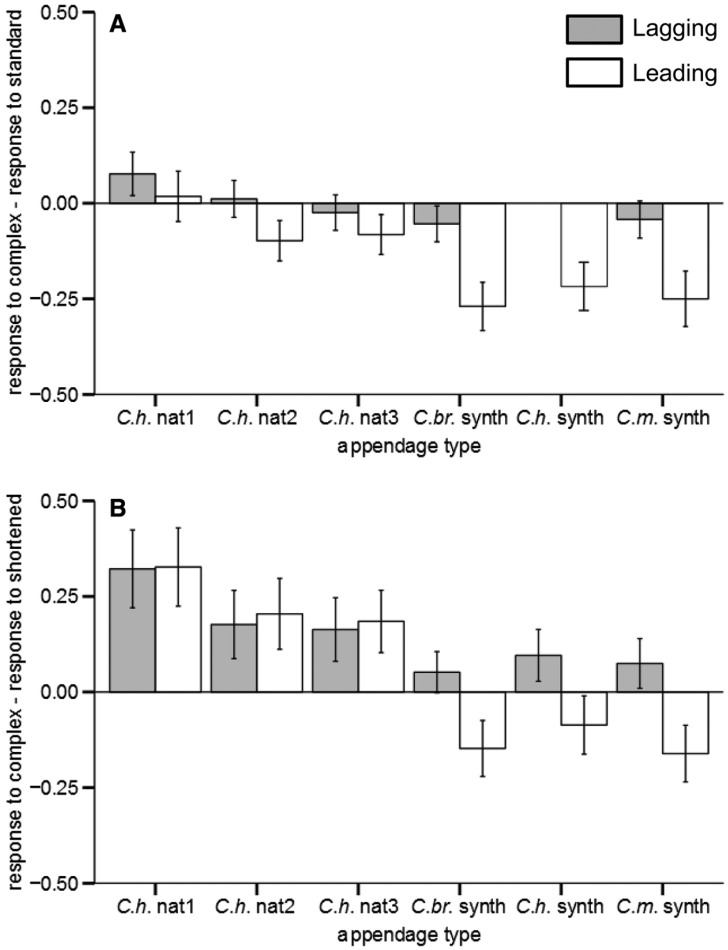

Figure 5. **Responses of females to complex songs with heterospecific appendages, appended to either the (A) standard or (B) shortened song stimulus. The appendage delay was 200 ms. Six different heterospecific appendages were used: three exemplars of natural *C. b. hedickei* appendages ("*C.h.* nat1-3") and three synthetic ("synth") appendages that resembled portions of the calling songs of either *C. brunneus* ("*C.br.*") or *C. mollis* ("*C.m.*"), or the appendage naturally produced by *C. b. hedickei* ("*C.h.*"). Each bar shows the mean (± 95% confidence interval) of the differences between each female's response to the given complex stimulus and its response to the (A) standard song or (B) shortened song, which had no appendage. Sample sizes are given in Table S1. Female responses could not be measured for the lagging synthetic *C. b. hedickei* + standard song stimulus (see Methods).**

less attractive and the third was neutral (Fig. 5A). One of the lagging *C. b. hedickei* exemplars had a positive effect on the attractiveness of the standard song, while the other two were neutral (Fig. 5A). In contrast, for the shortened song, both leading and lagging natural *C. b. hedickei* appendages had positive effects on song attractiveness (Fig. 5B). None of the heterospecific appendages elicited a strong response from females when presented without an accompanying song, although females responded nearly twice as often to one of the natural *C. b. hedickei* appendages as they did to any other appendage type (Fig. S16).

OVERALL EFFECTS OF TEMPORAL POSITION

We combined results across Experiments 1, 2, and 4 to further examine the effect of temporal position on the attractiveness of complex songs (Table 2). For standard songs, there was only one instance of a leading appendage with a positive effect, and neutral and negative effects were about equally as common. Most lagging appendages to standard songs were neutral, but a substantial portion had positive effects and there were only two instances of negative effects. For shortened songs, leading appendages most often had negative effects on song attractiveness, but there were some instances of positive effects. With only one exception, lagging appendages always had positive effects on the attractiveness of shortened songs (Table 2).

Discussion

Our function-valued analyses allowed us to characterize the adaptive landscape potentially guiding signal evolution by sexual selection, and to test alternative scenarios for the origin of complex signals under the sensory bias hypothesis. We found

Table 2. Combined results across all experiments for the effects of appendage position on the attractiveness of complex songs relative to the corresponding song without an appendage.

Standard song, noise appendages				Shortened song, noise appendages			
	Positive	Neutral	Negative		Positive	Neutral	Negative
Leading	1 (1.2%)	43 (51.2%)	40 (47.6%)	Leading	0 (0%)	2 (40%)	3 (60%)
Lagging	19 (22.6%)	64 (76.2%)	1 (1.2%)	Lagging	5 (100%)	0 (0%)	0 (0%)
Standard song, heterospecific appendages				Shortened song, heterospecific appendages			
	Positive	Neutral	Negative		Positive	Neutral	Negative
Leading	0 (0%)	1 (16.7%)	5 (83.3%)	Leading	3 (50%)	0 (0%)	3 (50%)
Lagging	1 (20%)	3 (60%)	1 (20%)	Lagging	5 (83.3%)	1 (16.7%)	0 (0%)

Appendages were defined as having positive, neutral, or negative effects when the mean response difference between the complex and simple song was positive and the confidence interval did not cross zero, the confidence interval crossed zero or the mean response difference was negative and the confidence interval did not cross zero, respectively. We tabulated these values separately for shortened and standard songs, and noise and heterospecific appendages, to illustrate the effects of these variables; the percentage of stimuli for the given stimulus type and appendage position that had each effect is given in parentheses.

hidden preferences for only a very limited set of complex acoustic signals in *C. b. biguttulus*; in many more cases the novel complex song was less attractive than the standard. Thus, there was no evidence for a general sensory bias for increased signal complexity per se (Ryan and Keddy-Hector 1992). Nevertheless, we were able to identify some patterns in the appendage characteristics that determined which complex songs were more attractive than the simple song currently produced by *C. b. biguttulus*, supporting a sensory bias scenario in which there are rules governing the types of appendages favored by hidden preferences. These patterns can be generalized to predict the potential evolutionary trajectories of complex signal evolution in this species. The temporal characteristics of novel signal elements were critical in determining the attractiveness of the resulting complex song. Furthermore, the strength and direction of these effects depended on characteristics of the main calling song. There is substantial between-species variation in the permissiveness of receivers (i.e., in the width of the preference function around its peak) in responding to variation in characteristics of complex signals (Wilczynski et al. 1999; Gerhardt et al. 2007a,b; Ryan et al. 2010; Seeba et al. 2010). The factors responsible for this variation are poorly studied, yet this is critical for understanding the evolution of diversity in animal signaling systems. Further investigations of the potential for complex signal evolution across diverse communication systems, when possible using a function-valued approach, are therefore badly needed.

BIASES AND LIMITS ON COMPLEX SIGNAL EVOLUTION

The trajectory of future signal evolution is in part a product of past evolutionary processes that selected for the communication system's current architecture (Ryan and Cummings 2013). In sexually selected signaling systems, studies have focused on the processes leading to the elaboration of signals, and the sensory bias hypothesis has been particularly influential in explaining how a species' past evolutionary history can result in biases that are or could be exploited by present-day signalers (Ryan 1998; Phelps and Ryan 2000; Ryan et al. 2001). The basic logic behind the sensory bias hypothesis can also be applied to explain why signal elaboration is sometimes limited. Past selection on the mechanisms by which sensory systems discriminate among different types of stimuli, detect signals in complex, noisy environments, and overcome the inherent limitations of nervous system morphology on stimulus processing all interact to shape the adaptive landscape of complex signal evolution and likely result in limits to the types of novel signal elements that may evolve (Ryan et al. 2001).

The most pronounced effects of adding acoustic appendages to male songs found here were negative: most long-duration, leading appendages made songs significantly less attractive. In contrast, lagging appendages were sometimes more attractive and otherwise neutral (Table 2). Furthermore, the slope of the preference functions indicates that the absolute magnitude of the strength of selection on appendage duration is greater for leading appendages than for lagging appendages. The differences between stimuli with leading and lagging appendages are noteworthy because both types of signal are equally complex and have equal sound energies. Therefore, female preferences for complex signals cannot be explained by their increased sound energy or by the simple fact that such songs are more complex. Instead, we identify strong temporal order effects, in which the same appendage was either attractive or unattractive depending on whether it was lagging or leading, respectively. Female gray treefrogs, *Hyla versicolor*, also discriminated against most complex songs with leading appendages and were attracted to some complex songs with lagging appendages (Gerhardt et al. 2007a; Henderson and

Gerhardt 2013), although there were weak, if any, temporal order effects in the closely related *H. chrysoscelis* (Seeba et al. 2010). Neither treefrog species produces complex calls in nature. In addition, female *Engystomops pustulosus*, a species in which males naturally produce complex calls, responded more strongly to complex signals with lagging appendages than to those with leading appendages, although signals with leading appendages were still highly attractive (Wilczynski et al. 1999).

The accumulated behavioral evidence, although limited, suggests that sensory systems tend to be biased against leading song elements that do not have species-typical characteristics, whereas these same elements may be irrelevant or even attractive when lagging if evaluation becomes more permissive across the signal's duration. We note, however, that sensory biases are not the only potential explanation for the evolution of complex signals or temporal-order rules. For instance, in many species, leading components of complex signals function as an alerting mechanism, increasing the likelihood that receivers attend to subsequent signal components, and in some cases changing the salience of those components (Hauber et al. 2001; Ord and Stamps 2008). Nevertheless, for *C. b. biguttulus*, we speculate that the challenges inherent in evaluating the fine-temporal structure of male songs with a small set of auditory neurons may have selected for a sensory architecture biased to find only certain kinds of signal elaborations attractive, while many others are highly unattractive. Female evaluations of song attractiveness in *C. b. biguttulus* are more influenced by unattractive than by attractive song elements, and the negative influence of unattractive elements is strongest at the beginning of the song (Clemens et al. 2014). Because the appendage stimuli were unattractive when presented alone, the female song evaluation system may therefore be especially biased against leading appendages. Long-duration leading appendages were especially unattractive. This may be because neuronal adaptation, which improves the processing of temporal characteristics as the signal progresses, has been shown to occur by 100–165 ms after the onset of signal reception in male *C. b. biguttulus* (Ronacher and Hennig 2004; Hildebrandt et al. 2015). If adaptation operates on a similar timescale in females, then portions of leading appendages longer than 100 ms would be especially well-processed by the female's sensory system, and thus potentially exert strong negative effects on song attractiveness. Therefore, previous selection for efficient processing of the temporal structure of male calling songs may be responsible for the rules governing the attractiveness of novel complex songs in *C. b. biguttulus*.

An exception to the temporal order effect described above occurred in some cases when complex songs contained natural appendages of the closely related *C. b. hedickei*. When a shortened *C. b. biguttulus* song was combined with a natural *C. b. hedickei* appendage, songs with both leading and lagging appendages were more attractive than the shortened song alone (Fig. 5B). This was not because the *C. b. hedickei* appendages themselves were especially attractive: females responded to such appendages infrequently when presented alone. Furthermore, when natural *C. b. hedickei* appendages were added to the standard *C. b. biguttulus* song we again found a difference in the attractiveness of leading- and lagging-appendage stimuli (Fig. 5A). Despite these qualifications, the effectiveness of *C. b. hedickei* appendages as additions to shortened *C. b. biguttulus* songs is significant because of the close evolutionary relationship between the two taxa. While the exact history is unknown, if the two groups are only recently diverged and if *C. b. hedickei* evolved complex songs from a simpler version, then females of the two subspecies may share common sensory biases. Similar exploitation of preexisting biases has been proposed to account for the evolution of complex signals, and predicts the responses of females to novel signal variation, in a diverse range of animals (Ryan and Rand 1993; Basolo 1995; Christy et al. 2003). Our identification of rules that govern complex song attractiveness in *C. b. biguttulus* also allows us to turn this hypothesis on its head to ask whether the same rules may have influenced the evolution of complex signals in *C. b. hedickei*. Specifically, it would be interesting to test whether female *C. b. hedickei* also find male songs with leading appendages unattractive. Many species in the genus *Chorthippus* produce songs with multiple elements (Ragge and Reynolds 1998), and temporal order effects have been identified in *C. dorsatus* (Stumpner and von Helversen 1992). Comparative analyses of the effects of appendages on song attractiveness in this and other species groups that vary in signal complexity will provide important insights into the extent to which signal diversification is explained by shared sensory constraints and biases.

DOES COMPLEXITY RESTORE SONG ATTRACTIVENESS?

The addition of novel signal elements could be a means of restoring the attractiveness of songs that otherwise are ineffective in eliciting responses from females. If "rescue" effects of appendages are common, this could provide an alternative path to the production of attractive signals, which may be exploited by certain classes of signalers and therefore lead to within-species signal diversification. We predicted that the positive effects of adding an acoustic appendage would be especially pronounced for less attractive versions of the simple song. This prediction received some support from comparisons of the effects of appendages on the standard song and on the less-attractive shortened song. Lagging appendages always improved the attractiveness of shortened songs, but only sometimes had an effect on the standard song. Leading appendages were usually unattractive for both song types. Lagging appendages may have been attractive additions to shortened songs because of the resultant increase in song duration.

However, although we found a large difference in female response to the simple versions of the shortened and standard songs, other studies found relatively weak preferences for song duration in *C. b. biguttulus* (Ronacher and Stange 2013). In contrast, songs with silent gaps inserted within syllables are highly unattractive to females (von Helversen 1972, 1979; Kriegbaum 1989; von Helversen and von Helversen 1997) and we found that they remained so even with the addition of noise appendages. The presence of gaps in songs is critical for discriminating males from females, and healthy males from injured males (von Helversen and von Helversen 1997).

The contrasting effects of appendages on the attractiveness of shortened and gappy songs suggest that certain types of song evaluation may be more susceptible than others to the effects of novel signal elements. Appendages may be at best irrelevant to critical discriminations of species or sex based on fine characteristics within individual signals (e.g., gappy songs). Judgements of signaler quality based on a signal's overall energetic content (e.g., song duration) may be more open-ended and therefore favor novel complex signals. Thus, in addition to the temporal order rules described above, we identified a second set of rules in which the attractiveness of complex signals depends on the interaction between appendage characteristics and characteristics of the base signal. This finding raises an important point, because the majority of our tests were performed with a single base song unit, the standard song. This stimulus was a synthetic representation of a typical male song and does not raise the concerns of pseudoreplication that are associated with the use of natural song recordings (McGregor 1992). Nevertheless, we cannot determine whether we would have obtained similar results for songs varying in other stimulus dimensions not tested here. Female preference function shape varies across many different song dimensions (Reichert and Ronacher 2015). An important focus of future research effort should involve extending the function-valued approach to examine the stability of the adaptive landscape of complex signal evolution across preexisting variation in male song characteristics. In general, such appendage-signal interactions have received very little attention (see also Henderson and Gerhardt 2013) but are likely to be of critical importance in the evolution of signal complexity because they potentially affect the variation in fitness of both signalers and receivers depending on preexisting costs and benefits of producing and responding to different types of signals.

Conclusions

Animal signals are inherently complex phenotypes, and studies of the evolution of signal complexity are important both because signals are excellent models of phenotypic evolution (Bradbury and Vehrencamp 1998), and because signal architecture plays a major role in diversification (Endler 1992; Wilkins et al. 2013; Schaefer and Ruxton 2015). Our measurement of female preference functions for novel complex songs identified some rules for when sensory biases will favor increased complexity, but we also found evidence that sensory biases sometimes limit complex signal evolution. These limits will influence the types of signal architectures that evolve, and may have been important in the evolution of song diversity in grasshoppers. Additional studies using a function-valued approach to estimate the adaptive landscape of sexual selection on signal structure across taxa with signals of varying degrees of complexity are badly needed to confirm the generality of the patterns of preferences for complex signals currently identified in a small group of species. Although acoustic signaling in grasshoppers is especially well-suited to this effort, similar approaches to the methodology described here can and should be applied to other taxa and signaling modalities. Another important extension is to examine the complexity of the entire signaling phenotype (Hebets et al. 2016). Grasshoppers, like many species (Candolin 2003; Higham and Hebets 2013), have multimodal courtship displays involving not only long-distance songs, but also close-range acoustic, visual and chemical signals (Vedenina and von Helversen 2003; Finck et al. 2016). Sensory biases may have been involved in the evolution of complex courtship, and it will be interesting to determine whether rules based on temporal order, spatial relationships and other characteristics determine the effectiveness of multiple components of the male signal repertoire on female mate choice. Understanding the causes and consequences of receiver responses to variation in novel signal structures is essential for understanding the evolution of diversity in animal communication systems.

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M.S.R. conceived of, and, along with B.R., designed the experiment, M.S.R. and J.F. performed field collections, experimental procedures and compiled raw data, M.S.R. analyzed data, M.S.R. wrote the paper with editing by B.R. and J.F.

DATA ARCHIVING

The doi for our data is doi:10.5061/dryad.51g4t/1.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1: Description of all of the experimental stimuli.
Table S2: Details of the natural *C. b. hedickei* appendage stimuli used in Experiment 4.
Table S3: Results of generalized linear mixed models testing effects of appendage and song characteristics on the absolute proportion of response to each stimulus.
Figure S1: Responses of females in Experiment 1 to complex songs with noise appendages of varying duration, appended to the standard song stimulus with a delay of 8 ms.
Figure S2: Responses of females in Experiment 1 to complex songs with noise appendages of varying duration, appended to the standard song stimulus with a delay of 32 ms.
Figure S3: Responses of females in Experiment 1 to complex songs with noise appendages of varying duration, appended to the standard song stimulus with a delay of 64 ms.
Figure S4: Responses of females in Experiment 1 to complex songs with noise appendages of varying duration, appended to the standard song stimulus with a delay of 100 ms.
Figure S5: Responses of females in Experiment 1 to complex songs with noise appendages of varying duration, appended to the standard song stimulus with a delay of 200 ms.
Figure S6: Responses of females in Experiment 1 to complex songs with noise appendages of varying duration, appended to the standard song stimulus with a delay of 300 ms.
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