



Motorway age is linked to larger body size, but not song carrier frequency, in male grasshoppers from roadside populations

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Abstract

Producing calls with increased minimum carrier frequency is well documented in sound communicating species confronted with noise pollution, possibly helping them to avoid acoustic masking of low-frequency signals. However, the lack of studies on invertebrates limits our understanding of the potentially adverse effects of anthropogenic noise on natural populations. While males of the grasshopper *Chorthippus biguttulus* are known to produce courtship signals with elevated low-frequency local maxima (LFLM) in roadside populations, previous studies provided no insight into morphological traits underlying LFLM shifts. Although developmental plasticity was identified as one main factor accounting for elevated LFLM, an additional role for a more hard-wired trait architecture was proposed, possibly as a result of adaptation to traffic noise. Our current study therefore aims to assess (1) whether the position of LFLM is related to male body size, (2) whether body size is correlated with motorway age, and (3) whether the position of LFLM is correlated with motorway age in *C. biguttulus* males from roadside populations. In addition to substantial variation in LFLM among *C. biguttulus* males, we found that larger males produced signals with lower LFLM, suggesting a potential role in female choice as a predictor of male quality. While we found no significant correlation between LFLM and motorway age, *C. biguttulus* males were larger near older motorway sections. This positive correlation may be due to several reasons, including: (1) higher nutrient concentration and availability in plant tissues due to eutrophication of roadside habitats; (2) temporal succession of male size phenotypes during colonization processes; and/or (3) selection for larger body size in roadside populations. Our results highlight the multifaceted and often complex nature of the relationships between morphology, signal traits and anthropogenic pressures on sound communication in invertebrates.

Keywords Acoustic communication · Anthropogenic change · *Chorthippus biguttulus* · Motorway age · Road noise · Structural body size

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Introduction

The influence of anthropogenic noise on animal communication is today recognized as a major issue from the perspectives of both behavioural and soundscape ecology, as well as conservation biology (Francis et al. 2009; Morley et al. 2013). Road noise is among the main factors leading to a decline in wildlife diversity near roads (McClure et al. 2013), with its adverse effects sometimes extending to more than 4 km from the source (Parris et al. 2009). Since road noise mainly falls into the lower part of the frequency spectrum, acoustic signals containing low frequency components are especially prone to acoustic masking (Slabbekoorn and Peet 2003; Francis et al. 2011) and many sound communicating species are thus potentially vulnerable to road noise pollution (McClure et al. 2013; Gurule-Small and Tinghitella 2018). Interestingly, a growing number of studies provide evidence for animals calling at a higher pitch when faced with anthropogenic noise, including traffic noise (Slabbekoorn and Peet 2003; Parris et al. 2009; Dowling et al. 2012). The vast majority of publications on this topic, however, concentrate on vertebrates (Francis et al. 2009; Parris et al. 2009; Nemeth and Brumm 2010); only a handful of studies addressed the impact of anthropogenic noise on invertebrates (Lampe et al. 2012, 2014; Shieh et al. 2012; Bunkley et al. 2017; Gurule-Small and Tinghitella 2018), in vast disproportion to the global abundance and general importance of invertebrates in terrestrial ecosystems (Morley et al. 2013).

Orthopteran insects are among the best studied sound communicating insect groups (Robinson and Hall 2002) and their acoustic signals, which serve to attract mates from a distance in most species, are particularly prone to signal degradation (Einhäupl et al. 2011; Schmidt and Balakrishnan 2015). Thus, orthopterans are highly suitable model organisms for studying the impact of anthropogenic noise on sound production, perception and the use of acoustic signals in attracting, locating and courting potential mates (Robinson and Hall 2002; Costello and Symes 2014; Bent et al. 2018; Gurule-Small and Tinghitella 2018). The bow-winged grasshopper *Chorthippus biguttulus* (Linnaeus, 1758) is a common European species and there is comprehensive knowledge of its sound production mechanisms, acoustic communication, female mating preferences and resulting sexual selection on male song traits (e.g. Ronacher et al. 2000; Safi et al. 2006; Stange and Ronacher 2012a, b). In this species, we find a bidirectional communication system, where male courtship songs are stimulating a female response song, which in turn triggers male response song and ultimately leads to a more close-range courtship behaviour (von Helversen and von Helversen 1997; Klappert and Reinhold 2003; Finck et al. 2016). A study by von Helversen and von Helversen (1997) suggested the specific importance of low-frequency local maxima (LFLM) of male *C. biguttulus* songs, occurring in the range of 6–8 kHz (von Helversen et al. 2004), in either species recognition or sex recognition. However, since traffic noise is known to reach amplitudes of 55–65 dB in this frequency range (Sandberg et al. 2003), LFLM is also especially prone to acoustic masking (Lampe et al. 2012, 2014).

In a previous study, Lampe et al. (2012) found that *C. biguttulus* males from roadside habitats produced courtship signals with elevated LFLM compared to males from non-roadsides habitats, presumably to avoid acoustic masking. The observed difference is unlikely to result from within-individual behavioural plasticity, since carrier frequency in *C. biguttulus* is most likely determined by fixed structural traits (Lampe et al. 2012). In fact, differences in carrier frequency among males from roadside and non-roadsides populations were stable for days after they were removed from their natural habitats (Lampe et al. 2012). The plasticity of structurally determined signal traits, such as amplitude and

carrier frequency (Montealegre-Z 2009; Einhupl et al. 2011), is generally presumed to be subject to developmental constraints in orthopterans (Ferguson 2018). Furthermore, stridulation itself is considered a neuronally hard-wired behaviour, leaving little room for individual short-term signal modification in response to prevailing environmental conditions (Einhupl et al. 2011). Although Lampe et al. (2012) found that heavier *C. biguttulus* males tended to produce louder signals with lower LFLM, structural body size had not been measured. Nevertheless, a close relationship between morphological properties of the stridulatory apparatus (typically correlated with body size) and signal traits is well documented in a number of sound communicating insect taxa (Bennet-Clark 1998; Garca-Navas et al. 2017). It is therefore vital to gain a better insight into morphological traits underlying the differences in LFLM, which was missing from Lampe et al. (2012).

In a follow-up study, Lampe et al. (2014) experimentally tested the effects of the acoustic environment during nymphal development on male *C. biguttulus* song traits based on a common garden approach, with experimental animals originating from either roadside or non-roadside populations exposed to differential noise levels. The results demonstrated that developmental plasticity accounted for part of the elevated LFLM in males reared under noisy conditions. However, a population-of-origin effect (roadside *versus* non-roadside) was evident as well; *C. biguttulus* males from roadside habitats produced higher LFLM than their non-roadside counterparts reared in the same conditions (Lampe et al. 2014), independently corroborating results from Lampe et al. (2012). In addition, when the data set from Lampe et al. (2012) was explored with regard to motorway age, a tendency towards higher LFLM was recorded in *C. biguttulus* populations adjacent to older motorway sections (Lampe et al. 2012). Motorway age represents the period of time a motorway section has been continuously open for traffic, i.e. the period of time a roadside population has been continuously exposed to traffic noise. These findings led the authors to suggest adaptive genetic differentiation as a possible explanation for the population-of-origin effect. However, the tendency found by Lampe et al. (2012; see above) was based on recordings from a sample of only eight *C. biguttulus* populations, with unequally distributed ages of adjacent motorway sections; three had been built in late 1930s, two in 1960s, two in 1980s and one in 2001. Hence, it is difficult to draw any firm conclusions from this previous study, without specifically and a priori addressing the relationship between noise-affected signal traits in roadside populations and motorway age.

Following up on Lampe et al. (2012, 2014), in the current study, we tested (1) whether among-male variation in singing performance is caused by differences in morphology, by assessing the relationship between structural body size (measured as pronotum and mean hind femur length) and LFLM in *C. biguttulus* males from 19 roadside populations. In orthopterans, larger individuals generally produce lower frequency calls than smaller ones (Brown et al. 1996; Montealegre-Z 2009; Montealegre-Z et al. 2017); thus, we would expect carrier frequency to be inversely proportional to body size in *C. biguttulus* males. Furthermore, we strategically sampled *C. biguttulus* males from the 19 populations adjacent to motorway sections of different age. We tested (2) whether body size of *C. biguttulus* males from the sampled roadside populations is related to motorway age, as this relationship might influence the relationship between LFLM and motorway age. Finally, we tested (3) whether the position of LFLM is related to motorway age in *C. biguttulus* males from the sampled roadside populations. Supposing elevated LFLM is favoured under noisy conditions, as indicated by the previous findings (Lampe et al. 2012, 2014), higher values of this trait would be an expected outcome of directional selection over a longer period of time. Thus, if adaptation to traffic noise has indeed occurred in *C. biguttulus*, on average higher LFLM would be expected in the populations adjacent to older motorway sections.

Materials and methods

Sampling and housing of grasshoppers

Adult *C. biguttulus* males were sampled from 19 roadside populations, located in Western, Northern, Southern and Central Germany, between the end of July and late September 2015. Sampling locations were situated near motorway sections that had been opened for traffic between 1936 and 2006 (Table 1). We used meadows at rest stops as sampling locations. Locations were selected so that they were at least 30 km from each other and widely distributed over different motorway sections varying in the time since they had been opened to traffic. We sampled grasshoppers one to four meters from the road, detecting them visually and catching them by hand. Altogether 244 adult males (12–25 per location) and about 60 adult females (a few per location, used to stimulate males during the recording process, see below), were collected and transferred to 29 °C climate chambers at Bielefeld University. Prior to song recording, we kept the males from the same location collectively but separate from females in cages (size 20×20×30 cm) and fed them *ad libitum* with a mixture of grasses (Poaceae). After the recording process was completed, each male was weighed using a Sartorius L220S analytical scale, killed by freezing, labelled and preserved at room temperature in 96% ethanol in a microtube for further examination.

Table 1 Sampling locations of male *Chorthippus biguttulus* grasshoppers, including their geographic coordinates, the adjacent motorway, the year when the respective motorway section was opened for traffic and the number of sampled individuals (N)

Location	Geographic coordinates		Motorway	Year opened	N
	Latitude	Longitude			
Rastplatz Rohrse Süd	52.360064	10.155787	A2	1936	11
Altenburger Land near Schmölln	50.863408	12.321242	A4	1937	10
Wolfshöhe	49.544797	11.320950	A9	1937	6
Rastplatz Brunsberg	51.724014	7.979823	A2	1938	13
Hörselgrau near Gotha	50.912628	10.587383	A4	1940	9
Raststätte Bühl	48.752731	8.130367	A5	1958–59	16
Hildesheimer Börde	52.126359	10.051856	A7	1960	13
Raststätte Haidt	49.779574	10.246865	A3	1964	13
Raststätte Im Mersch	51.773902	7.640275	A1	1965	12
Raststätte Mählerthshof	50.684857	9.682873	A7	1968	7
Raststätte Am Biggenkopf	51.489413	9.021141	A44	1971	20
Raststätte Brockbachtal	52.267281	7.906450	A30	1976	15
Am Sonnengau Scharfenstein Ost	51.195382	9.402609	A49	1976	16
Raststätte Hahnenkoppel	53.572572	10.301058	A24	1982	18
Raststätte Teutoburger Wald Ost	52.139485	8.144857	A33	1983	14
Raststätte Rotmaintal	50.033090	11.472491	A70	1991	6
Rastplatz Leinetal	51.401637	10.199356	A38	1999	15
Trebbetal Nord	54.087408	12.951766	A20	2002	15
Pröbber See West	53.810086	11.604647	A14	2006	15
Sum					244

Locations are ordered by the year the adjacent motorway sections were opened for traffic

Song recording and analysis

Male *C. biguttulus* song typically consists of several phrases 2–3 s long (von Helversen and von Helversen 1997) (Fig. 1a). Each phrase contains between 20 and 40 syllables, around 80 ms long, separated by short pauses (10–15 ms) (von Helversen and von Helversen 1997; Lampe et al. 2012). We obtained a total of 1361 song recordings (containing a single song each) from the 244 males, with 5–10 recordings per individual.

Recordings took place between 9 a.m. and 6 p.m. in a dark, sound-isolated chamber at 29 °C, one to five days after the transfer to captivity. During the process, we placed a male within a sound-proof wooden open box (112×100×69 cm), the inner surface of which was covered with pyramid foam to reduce uncontrolled ambient noise and reverberation. Since *C. biguttulus* is a diurnal, thermophile species, a focal male was placed upon a small illuminated patch in the middle of the box. To stimulate a male to call, we placed a randomly chosen female from the same population directly in front of the male. As grasshoppers exhibit phototactic responses to light sources (Kral 2008), the pair would remain on the illuminated patch during recording. Whenever a male started approaching a female for mating, or a female responded to a male's song, the observer would stop recording, and prevent mating. If the obtained song recording was too short, the recording procedure would subsequently be repeated.

Songs were recorded using a Bruel & Kjaer 4939 microphone (Naerum, Denmark 1/4 Microphone Free Field) with frequency response range from 4 to 100,000 Hz and

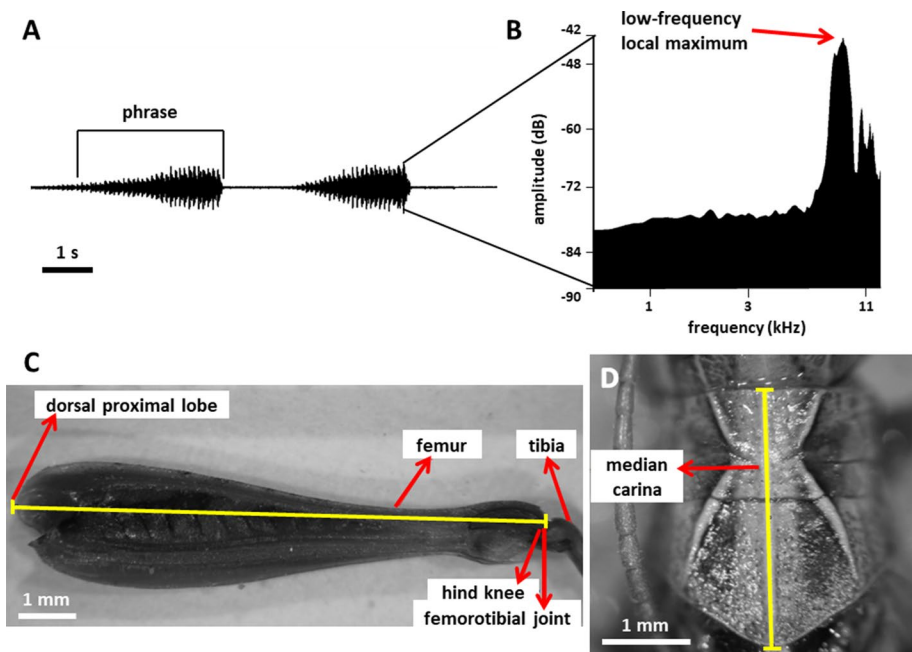


Fig. 1 **a** Temporal structure (amplitude over time) of a *Chorthippus biguttulus* song containing two phrases; **b** power spectrum of a *C. biguttulus* phrase, with the low-frequency local maximum located at 8428 Hz; **c** hind femur length and **d** pronotum length measurements (marked by yellow lines) taken from *C. biguttulus* males

microphone sensitivity of 4 mV Pa^{-1} . The microphone was placed on a tripod at 8 cm distance from a male, and was connected by NC*MX-HD cable connector (Neutrik, Charlotte, NC, USA) to a standard desktop PC. We used Avisoft-SASLab Lite bioacoustics software (Berlin, Germany, <http://www.avisoft.com/>), with a sampling rate of 250 kHz, to record male acoustic signals.

Songs were analysed in Audacity® software (version 2.1.3), using the option „Plot Spectrum“, with default size of 1024 samples and Hanning window function. We plotted frequency spectra for four phrases per song, except in cases when the song contained less than four phrases. The highest frequency peak in the 5.5–10.7 kHz range, which corresponds to LFLM of the signal, was taken for each phrase (Fig. 1b). In a portion of recordings (for 25 males), there were two or more LFLM peaks of the same height per phrase, potentially due to low quality of these recordings. We did not consider such recordings in the subsequent analyses. A single male had unusually high LFLM in all phrases, above 10.3 kHz, thus falling far off the 6–8 kHz range shown in von Helversen and von Helversen (1997) and von Helversen et al. (2004). Recordings of this male were excluded from the analyses. Overall, we successfully analysed song recordings of 218 males.

Morphometric analysis

In gomphocerine grasshoppers, both males and females produce acoustic signals by rubbing files of pegs on the inner sides of their hind femora against a thickened vein on the tegmina (von Helversen and von Helversen 1997; Gottsberger and Mayer 2007). Previous studies on *C. biguttulus* demonstrated correlations between song traits and size-related morphological traits of the stridulatory apparatus, such as the length of hind femora (Stange and Ronacher 2012a, b).

Accordingly, hind femora were carefully pulled out with a forceps and removed from the thorax of each specimen. We took photos of left and right hind femur (10x magnification), and pronotum (20x magnification), using a Canon EOS 600D camera connected to a stereomicroscope (Leica, M2125, type 376,788). Hind femur length was measured from the apex of dorsal proximal lobe to the apex of the hind knee (femorotibial joint) (Fig. 1c), whereas pronotum length was measured along the median carina in dorsal view (Fig. 1d). We quantified the morphometric traits using ImageJ 1.47v image processing and analysis software (Schneider et al. 2012).

To estimate repeatability of morphometric measurements, we took photos of hind femora and pronotum from 19 randomly selected males for a second time, and measured corresponding morphometric traits as described above, blind to the identity of the samples. Overall, we successfully analysed morphometric traits of 213 males (see also next paragraph).

Statistical analysis

We used R 3.5.3 (R Core Team 2018) for all computations. We used the R package *rptR* (Stoffel et al. 2017) to calculate the repeatability of repeated measurements of the same individual morphological structures based on linear mixed effects models and including 95% confidence intervals (parametric bootstrapping, $N = 10,000$ replicates). We fitted the grand mean of the respective trait as the only fixed effect and morphological structure identity as the only random effect. Repeatability of femur length was 0.36 (95% CI 0.09–0.59); mean femur length was used in all subsequent analyses.

Repeatability of pronotum length was 0.32 (95% CI 0.00–0.67). To obtain an overall measure of structural body size, we used the principal component 1 (PC1) from a principal component analysis of mean femur length and pronotum length. PC1 explained 75% of the variance in the data set.

Linear mixed effects models were fitted using the R function *lmer* (library: *lme4*; Bates et al. 2015) to test for relations between LFLM and structural body size and motorway age. In these models, structural body size and motorway age were considered as fixed effect explanatory variables, while song identity nested in individual identity nested in sampling location were included as random effects. To test for the effects of motorway age on structural body size, we considered motorway age as fixed effect explanatory variable, and sampling location was included as a random effect. Motorway age was mean-centered to obtain biologically relevant intercept estimates of LFLM and structural body size, respectively.

Following Nakagawa et al. (2017), we used the R package *MuMIn* 1.43.6 (Bartoń 2019) to compute marginal and conditional R^2 to estimate the proportion of variance explained by fixed effects or by both fixed and random effects combined, respectively. Significance of fixed effects was determined by *t* tests with Satterthwaite's approximation of degrees of freedom using the R package *lmerTest* 3.1-0 (Kuznetsova et al. 2017). All statistical tests were two-tailed and the null hypothesis was rejected at $p < 0.05$.

The 218 males with successfully analysed song recordings were considered in the statistical analysis of natural variation in LFLM, while all analyses involving morphometric measurements were restricted to 213 males with successfully analysed morphology (the identity of four collected specimens was ambiguous and one individual escaped after song recording but before collecting; song recordings obtained from these five individuals therefore had to be excluded from the latter set of analyses).

Results

Natural variation in low-frequency local maxima

LFLM were successfully analysed in 1665 phrases from 793 songs of 218 males originating from 19 locations. LFLM ranged from 5333 to 9283 Hz with an overall mean estimate of 7216 ($\pm$ SE 92) Hz (linear mixed effects model REML fit with the intercept as only fixed effect and song identity nested in individual identity nested in location as random effects; Table 2). We found random variation in LFLM among songs (within individuals, explaining 5.1% of the total variance), among individuals (within locations, 81.9%) and among locations (11.4%; see Table 2 for full model output including variance estimates).

Biophysical considerations lead us to expect a negative relationship between structural body size and song frequency. Furthermore, if in addition body size varies with motorway age, this may confound any analysis of LFLM in relation to motorway age. Therefore, we first analysed the relationship between LFLM and structural body size, and tested whether structural body size and motorway age may be correlated, before subsequently analysing the relationship between LFLM and motorway age.

Low-frequency local maxima in relation to structural body size

We analysed both LFLM and structural body size, the latter measured as the first principal component of a PCA based on pronotum length and mean hind femur length, for 1638

Table 2 Results of a linear mixed effects model of low-frequency local maxima (LFLM; Hz) for 1665 phrases of 793 songs of 218 *Chorthippus biguttulus* males originating from 19 locations

Fixed effects	Estimate $\pm$ SE	<i>t</i>	<i>df</i>	<i>p</i>
Intercept	7215.8 $\pm$ 92.0	78.43	15.90	<0.001
<i>Random effects</i>				
Variance		—	—	—
Song (intercept)	42,364	—	—	—
Individual (intercept)	683,523	—	—	—
Location (intercept)	94,889	—	—	—
Residual	13,739	—	—	—

The model included the intercept as only fixed effect (thus estimating the overall mean LFLM), while song identity, individual identity and sampling location were included as nested random effects (thus estimating natural variation in LFLM among songs, individuals and locations, respectively). The purpose of this null model was estimating the relative importance of variance components; thus, restricted estimation of maximum likelihood (REML) was used. Proportion of variance explained: Conditional $R^2 = 0.984$

phrases from 776 songs of 213 males originating from 19 locations. LFLM decreased with male structural body size ($p < 0.001$; Fig. 2; Table 3), but structural body size explained only 7.4% of the variance in LFLM (see also Table 3).

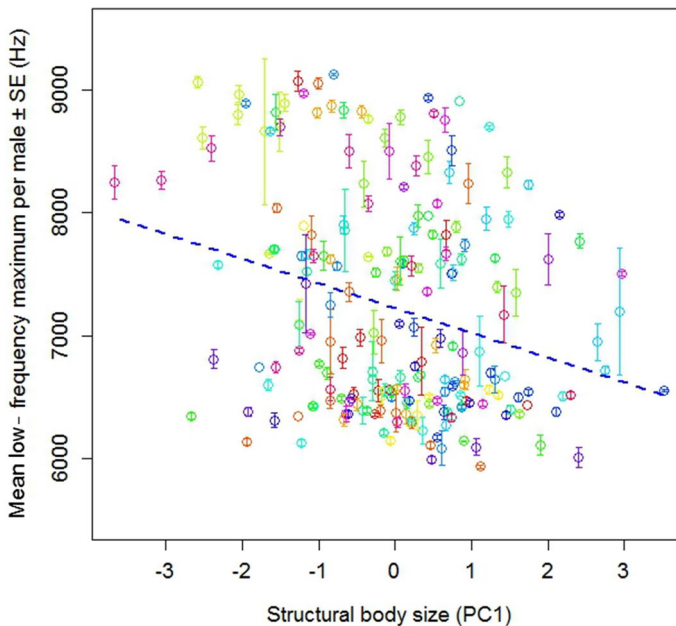


Fig. 2 Mean low-frequency maximum per male ($\pm$ SE) in relation to structural body size for $N = 213$ males from 19 roadside locations. Structural body size is represented by the first principal component derived from a principal component analysis based on pronotum length and mean femur length per male. Colours indicate location identity and the line represents the slope estimate from a linear mixed effects model with song identity nested in individual identity nested in location as random effects (see also Table 3)

Table 3 Results of a linear mixed effects model of low-frequency local maxima (LFLM; Hz) as a function of structural body size (PC 1 of pronotum length and mean hind femur length) for 1638 phrases of 776 songs of 213 *Chorthippus biguttulus* males originating from 19 locations

Fixed effects	Estimate $\pm$ SE	<i>t</i>	<i>df</i>	<i>p</i>
Intercept	7223.46 $\pm$ 90.98	79.39	16.93	<0.001
Structural body size	- 200.96 $\pm$ 49.04	- 4.10	211.04	<0.001
<i>Random effects</i>				
Variance		-	-	-
Song (intercept)	42,293	-	-	-
Individual (intercept)	607,460	-	-	-
Location (intercept)	97,255	-	-	-
Residual	13,027	-	-	-

The purpose of this model was estimating the significance of fixed effects; thus, maximum likelihood (ML) was used. Proportion of variance explained: Marginal $R^2=0.074$; Conditional $R^2=0.984$

Structural body size in relation to motorway age

Structural body size decreased significantly with the year the motorway section came under traffic ($p < 0.001$; Fig. 3; Table 4). Motorway age explained 14.4% of the variance in structural body size (see also Table 4).

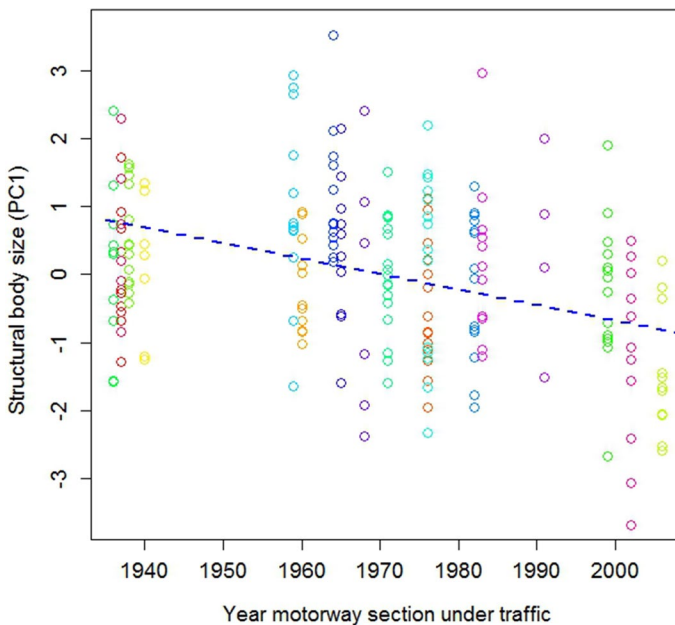


Fig. 3 Structural body size in relation to motorway age for $N=213$ males from 19 roadside locations. Structural body size is represented by the first principal component derived from a principal component analysis based on pronotum length and mean femur length per male. Colours indicate location identity and the line represents the slope estimate from a linear mixed effects model with location as random effect (see also Table 4)

Table 4 Results of a linear mixed effects model of structural body size (PC 1 of pronotum length and mean hind femur length) as a function of motorway age for 213 *Chorthippus biguttulus* males originating from 19 locations

Fixed effects	Estimate $\pm$ SE	<i>t</i>	<i>df</i>	<i>p</i>
Intercept	0.0005 $\pm$ 0.1038	0.005	18.57	0.996
Motorway age	− 0.023 $\pm$ 0.004	− 4.86	19.07	<0.001
Random effects	Variance	—	—	—
Location (intercept)	0.1866	—	—	—
Residual	1.0874	—	—	—

The purpose of this model was estimating the significance of fixed effects; thus, maximum likelihood (ML) was used. Proportion of variance explained: Marginal $R^2=0.144$; Conditional $R^2=0.269$

Motorway age was mean-centered

Low-frequency local maxima in relation to motorway age

Based on the significant relations reported above, we included structural body size as a covariate in the analysis of LFLM. Controlling for the additive effect of structural body size ($p<0.001$; Table 5), we found no evidence that LFLM was related to motorway age ($p=0.46$; Fig. 4; Table 5). The percentage of variance in LFLM explained by motorway age was 1.3% (see also Table 5).

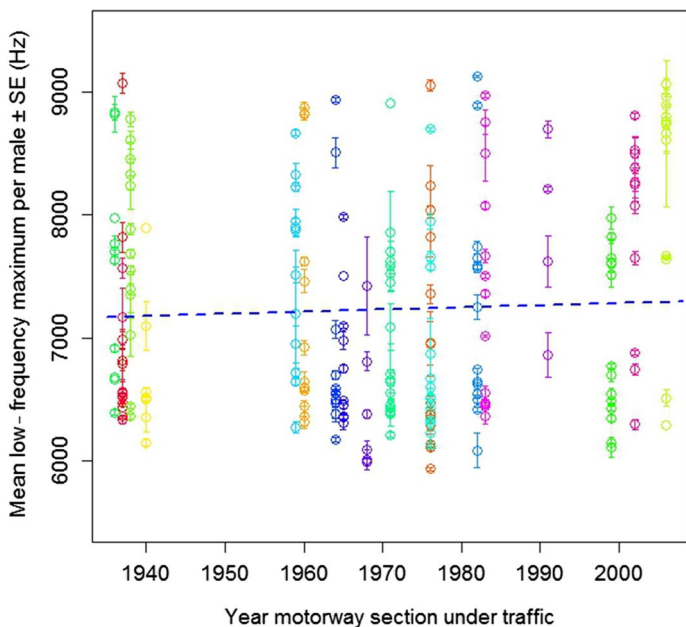


Fig. 4 Mean low-frequency maximum per male ($\pm$ SE) in relation to motorway age for $N=213$ males from 19 roadside locations. Colours indicate location identity and the line represents the slope estimate from a linear mixed effects model with song identity nested in individual identity nested in location as random effects while simultaneously controlling for the fixed effect of structural body size (see also Table 5)

Table 5 Results of a linear mixed effects model of low-frequency local maxima (LFLM; Hz) as a function of motorway age and structural body size (PC 1 of pronotum length and mean hind femur length) for 1638 phrases of 776 songs of 213 *Chorthippus biguttulus* males originating from 19 locations

Fixed effects	Estimate $\pm$ SE	<i>t</i>	<i>df</i>	<i>p</i>
Intercept	7229.01 $\pm$ 90.80	79.6	17.25	<0.001
Structural body size	− 193.41 $\pm$ 50.00	− 3.87	212.00	<0.001
Motorway age	3.15 $\pm$ 4.20	0.75	20.13	0.46
<i>Random effects</i>				
Variance		—	—	—
Song (intercept)	42,292	—	—	—
Individual (intercept)	606,304	—	—	—
Location (intercept)	95,691	—	—	—
Residual	13,027	—	—	—

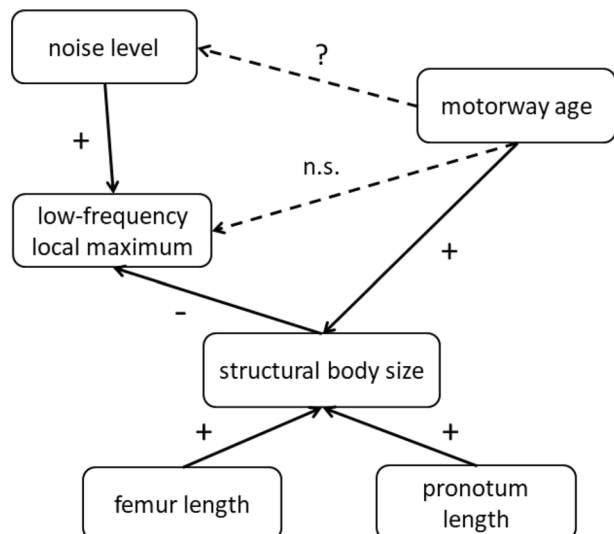
The purpose of this model was estimating the significance of fixed effects; thus, maximum likelihood (ML) was used. Proportion of variance explained: Marginal $R^2=0.087$; Conditional $R^2=0.984$

Motorway age was mean-centered

Discussion

In line with predictions from biophysics and the results of previous studies (Bennet-Clark 1998; Stange and Ronacher 2012a, b; Montealegre-Z et al. 2017), the current study demonstrates that structurally larger *C. biguttulus* males produce signals with lower LFLM than smaller ones. Our results also show a strong positive relationship between motorway age and male body size, i.e. the younger the motorway section, the smaller the mean body size in directly adjacent *C. biguttulus* populations. Finally, controlling for the effects of body size, we found no correlation between motorway section age and the position of LFLM in roadside populations of this species. The main relationships investigated in this study and the previous studies on *C. biguttulus* by Lampe et al. (2012, 2014) are summarized in Fig. 5.

Fig. 5 Schematic diagram summarizing the relationships investigated in the current study and previous studies by Lampe et al. (2012, 2014); significant relationships are represented by solid lines, whereas non-significant or unknown relationships are represented by dashed lines; +, positive relationship; −, negative relationship; n.s., non-significant; ?, unknown relationship



In orthopterans, signal traits correlated to body size are often used by females as honest indicators of male quality (Brown et al. 1996; Klappert and Reinhold 2003; Lehmann and Lehmann 2008). Body size correlates strongly with body condition and fitness (Whitman 2008; Stange and Ronacher 2012a, b), larger individuals typically having better energy reserves and being more resistant to starvation (Whitman 2008). Stange and Ronacher (2012a, b) found that certain song traits in *C. biguttulus*, e.g. syllable onset accentuation and offset, were positively correlated with both male size and immunocompetence. Our results suggest that LFLM might be another reliable predictor of male quality in *C. biguttulus*, with a potential role in female choice, as proposed by Lampe et al. (2012). This should be tested experimentally in future work. Body size, however, explained a relatively small percentage of the total variance in LFLM. This is not altogether surprising, as the morphological traits in *C. biguttulus*, although variable, are known to exhibit lower within-population variation than certain dynamic song traits (see below; Stange and Ronacher 2012a, b).

Furthermore, we propose three possible explanations for the novel finding of a positive relationship between motorway age and male body size in roadside *C. biguttulus* populations:

1. Motorway presence may change nutrient composition and availability in the roadside environment, which is reflected in the mean body size of *C. biguttulus* populations. Soil chemistry at roadside localities is known to be strongly affected by traffic density (Rodríguez-Flores and Rodríguez-Castellón 1982; Angold 1997; Loganathan et al. 2013). Vehicle exhausts and, likely, human urine deposition and defecation at the rest stations cause eutrophication of roadside habitats, adding nitrogen to the soil, which in turn enhances growth and overall above-ground productivity of vascular plants, particularly grasses (Angold 1997). Increased nitrogen concentration in plant tissues means higher food plant quality and nutrient availability for herbivorous insects. An increase in abundance is a well-documented response of some herbivorous insects, e.g. the lepidopterans *Phalera bucephala* and *Euproctis similis*, to road proximity (Port and Thompson 1980; Angold 1997). Furthermore, nutrition is known to influence body size in orthopterans (Whitman 2008). In particular, higher nitrogen content in food plants and its availability in the form of soluble proteins and amino acids result in faster growth and larger body size of *C. biguttulus* (Franzke and Reinhold 2011). Therefore, it seems plausible that an increase in mean body size due to nutrition could occur in roadside populations. However, the effect might become apparent only in older roadside populations, which have been exposed to the changed environment for a longer period of time. This issue could be clarified in future studies by looking for differences in nutrient concentration in plant tissues at localities adjacent to motorway sections of different age. Higher nutrient concentration would thereby be expected near older motorway sections, due to longer exposure of roadside vegetation to traffic.

2. There may be a temporal succession of male phenotypes during colonization events, as a result of differential success in intraspecific competition. In most orthopteran species, larger individuals are typically more successful in both inter- and intraspecific competition and accordingly have higher fitness (Joern and Klucas 1993; Whitman 2008). Due to competitive inferiority and female choice, smaller males often have decreased mating success and lower reproductive rate in comparison to larger ones (Stange and Ronacher 2012a, b). Nevertheless, being generally more mobile and agile (Whitman 2008), smaller individuals are more likely to disperse and colonize new habitats when exposed to strong competition. Thus localities adjacent to recently opened motorway sections might initially be colonized by smaller males, but a change of phenotypes may occur at a later stage, as larger males disperse from the source populations and outcompete smaller ones. Older and younger roadside populations may therefore represent different stages of this succession. If the

colonization process in *C. biguttulus* involves a systematic succession of male body size phenotypes in newly established populations, it should be possible to find a similar pattern in any recent colonization event. This could be tested with a long-term in situ experiment, in which mean male body size would be monitored across extended periods of time within newly established *C. biguttulus* population replicates.

3. Males may be selected for larger body size in roadside populations. It has been suggested that predation might decrease near motorways (Morley et al. 2013), as traffic noise impairs the ability of potential predators to locate prey using acoustic cues, decreasing their foraging efficiency (Siemers and Schaub 2011). Vertebrate predation in particular may be a strong size-selective force in orthopterans (Whitman and Vincent 2008). Predation rate generally increases with body size, larger individuals being more conspicuous, more rewarding energetically and often easier to catch (Whitman 2008; Whitman and Vincent 2008). However, if predation is lower, directional selection towards larger male body size could take place in roadside populations, due to intrasexual competition and female choice (see 2 above). The result of this selection process, i.e. increased mean body size of males, might become evident in older roadside populations, which have been exposed to a motorway long enough for a phenotypic change to occur. The potential role of predation-related selection could be tested in the future by monitoring survival rates of *C. biguttulus* males of different body sizes in population replicates experimentally exposed to different predation levels.

Ultimately, we cannot rule out the possibility that the increase in mean body size with motorway age occurs due to synergistic effect of two or all three processes discussed above. Motorway age, however, explained a relatively low percentage of the total variance in body size, but this is most likely due to high natural variation in body size within *C. biguttulus* populations, possibly reflecting environmental fluctuations in resource availability and microclimatic factors during development (Stange and Ronacher 2012a, b).

Contrary to our expectations, mean LFLM was not correlated with motorway age, but it was slightly higher in *C. biguttulus* males sampled near more recently built motorway sections, probably due to smaller mean body size within these populations (see above). However, body size is likely one of many traits, such as wing (tegmen) size, size of the sound-radiating wing cells etc. (not necessarily correlated with body size 1:1; see e.g. Montealegre-Z et al. 2017), that can influence the position of LFLM. The observed pattern could thus be a consequence of simultaneous selection for larger body size (see above) and higher LFLM (due to anthropogenic pressure, i.e. traffic noise) in roadside environment, as already suggested by Lampe et al. (2012). Alternatively, the strength of selection for higher LFLM may not be associated (only) with motorway age, since the motorway sections of similar age can have different noise levels (e.g. some sections being frequented more by trucks than by cars or differing in asphalt characteristics; see Gross et al. 2010; Shannon et al. 2016). However, LFLM may also be influenced by the parental environment through parental or epigenetic effects (Lampe et al. 2012, 2014). This is perhaps a more likely explanation for the population-of-origin effects found in Lampe et al. (2012, 2014) than the influence of early nymphal environment, since hearing organs in orthopterans, required for assessing the environmental noise level, are usually not developed prior to the third nymphal instar (Yager 1999; Kasumovic et al. 2011). Furthermore, grasshoppers might be selected against adjusting to the environmental noise level early on in their development, considering that the conditions may change substantially until they reach adulthood.

According to our findings, only 11.4% of the variance in LFLM of *C. biguttulus* male courtship songs can be explained by factors effective among locations, and most variance can be explained by variables varying within locations, with variation among individuals

explaining as much as 81.9% of the total variance. This may be at least partly explained by high among-individual variation in body size (see above). Nevertheless, studying male songs in four populations of *C. biguttulus*, Stange and Ronacher (2012a, b) found substantial within-population variation in most song parameters, including syllable to pause ratio, offset and onset. High among-individual variation is well documented for ‘motivational’ or dynamic signal traits, with females preferring the highest naturally occurring values of such traits to values at or near the population mean (Gerhardt 1991; Stange and Ronacher 2012a, b). Accordingly, unlike static signal traits, upon which stabilizing selection is exerted by female choice, dynamic traits tend to exhibit large differences among the males within a population (Gerhardt 1991). This could be the case with LFLM, too, a trait potentially influencing the attractiveness of male signals (see above).

Conclusions

This study confirms the importance of structural body size in predicting spectral properties of acoustic signals in orthopterans; in *C. biguttulus*, LFLM may play a role in female choice as a predictor of male quality. Although our results suggest that no adaptation of male acoustic signals has occurred in *C. biguttulus* populations exposed to traffic noise for a longer period of time, motorway presence did have a strong effect on male body size in such populations. Causes of the observed relationship are still unclear and need to be investigated by *in situ* studies in the future. Our findings highlight the multifaceted nature of the ecological impact of major roads upon roadside invertebrates, with many patterns and processes still insufficiently understood, offering numerous prospects for further research.

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Author contributions F.R. analysed the courtship songs and lead the writing of the manuscript. K.P. recorded the courtship songs and created the protocol for taking morphometric measurements. M.I. performed the morphometric measurements. K.R. proposed the project, lead the field work and supervised the bioacoustic analysis. T.S. performed the statistical analysis, contributed to writing and supervised the morphometric analysis. All authors commented on the manuscript draft and gave the final approval for publication.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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