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Causes of variability in male vibratory signals and the role of female choice in Mantophasmatodea

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ABSTRACT

Communication systems that involve substrate vibrations are increasingly a focus of research since this communication mode - recently termed biotremology - has been found to be remarkably widespread in the animal kingdom. Vibrational signals are often used during courtship and therefore underlie both natural and sexual selection. Mantophasmatodea use species- and sex-specific substrate vibrational signals during courtship. We explored whether male vibrational signals of the South African heelwalker *Karoophasma biedouwense* vary with temperature, body condition and age, and tested female preference towards various signal pattern combinations. We recorded male signals under varying temperatures and over 3.5 weeks after onset of signaling. Our results show that the temporal structure of male signals is modified by changes in temperature, and changes with male age. Other characteristics, especially duty cycles, are less affected, but correlate with body condition. Females responded along a broad spectrum of signaling patterns, indicating that they do not favor signals of males of a certain age or condition. They were selective towards the fine structure of vibratory signals, suggesting that pulse repetition times carry species-specific information. Mantophasmatodea thus use vibrational signals to identify and localize a mating partner, but presumably not for precopulatory mate selection.

1. Introduction

Biotremology investigates signals and cues transmitted via diverse substrates, focusing on surface-borne vibrational waves (Hill and Wessel, 2016). Arthropods have been shown to use substrate-vibrational communication signals for various reasons such as mate localization, species and sex recognition, predator and prey detection, and social interactions (e.g. Brownell and Farley, 1979; Cocroft and Rodríguez, 2005; Sullivan-Beckers and Cocroft, 2010; Virant-Doberlet et al., 2011; Hill and Wessel, 2016). The long evolutionary history of this communication modality, the remarkable diversity of species in which it occurs, and its role in biotic interactions provide unparalleled opportunities to ask and answer various general questions concerning species recognition, signal evolution, sexual selection, and the role of communication signals in population differentiation and speciation (Endler, 2014). Despite the extensive use of vibrational signals by animals, research on this topic is still underrepresented, and many aspects of this communication mode are in need of investigation (Hill and Wessel, 2016).

Detailed investigations on the evolution of insect vibrational communication signals exist for only a few taxa. For most of them, vibrational signals are related to sexual behavior and used for mate recognition and localization (Virant-Doberlet and Cokl, 2004). So far, the studies focus mainly on herbivorous Hemiptera (stink bugs, *Nezara viridula*, e.g. de Groot et al. (2011); treehoppers, e.g. Rodríguez and Cocroft (2006); planthoppers, e.g. Winter and Rollenhagen (1990); leafhoppers (Derlink et al., 2014)). These groups produce elaborate vibrational signals on their host plants by tremulation (i.e. vibrating parts of the body and transmitting vibrations to the substrate via the legs). The role of vibrational communication signals in species recognition and premating isolation has rarely been systematically studied. Moreover, the systems studied so far have revealed differences between them, namely signal variability, recognition abilities and preferences by females and males that differ considerably between the investigated taxa (Derlink et al., 2014).

Mantophasmatodea (Heelwalkers) are wingless predatory insects that use signals produced by drumming the abdomen onto the surface (viz. percussion), thus creating substrate vibrations of a species- and sex-specific pattern. The vibrational calls serve for sex identification and mate localization (Eberhard and Picker, 2008; Eberhard and Eberhard, 2013; Eberhard and Treschnak, 2018). Male and female calls can be distinguished by their temporal structure and complexity: Male

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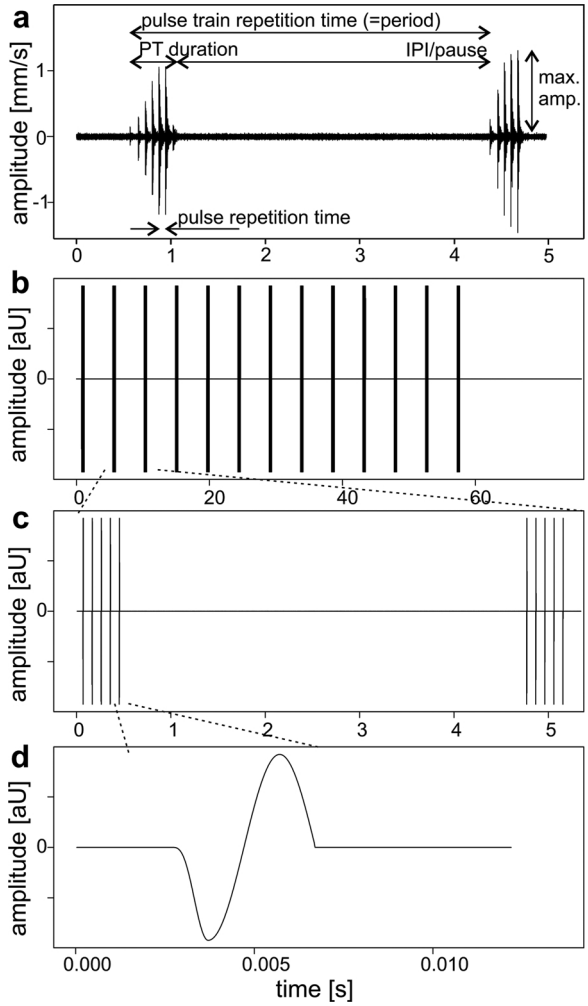

Fig. 1. a) Vibrational communication signals of a *K. biedouwense* male, measured with a Laser-Doppler vibrometer, showing two pulse trains and the variables measured and varied for the playback tests. b–d) Artificially generated vibratory playback signals at different timescales with b) showing a full playback test of one minute, c) showing two pulse trains with 5 pulses each, and d) showing one single pulse within a pulse train.

signals consist of repeated groups of pulses (pulse trains - PT; Fig. 1a), while the simpler female signals comprise repeated single pulses. So far, the structure of the vibrational signals has been investigated in 15 species; the examined taxa differ in several parameters of the calls (Eberhard and Eberhard, 2013; Roth et al., 2014; Conti et al., 2019). This is surprising given that most of the species are allopatric and no overlapping of species boundaries is known for most of the populations (Klass et al., 2003; Wipfler et al., 2018). The only detailed behavioral analysis on heelwalker vibratory communication so far used two sympatric Austrophasmatid taxa, *Karoophasma biedouwense* Klass et al., 2003 and *Viridiphasma clanwilliamense* Eberhard et al., 2011: Females reacted with continuous tapping and ceased locomotion when presented with signals of conspecific mates, while they did not tap and increased locomotor activity when subject to the playback of the heterospecific species (Eberhard and Picker, 2008).

The basis of all investigations concerning the evolution of substrate-borne vibrational signals relies on the phenotypic variation of the respective signals, both between and within individuals. Causes for such variability can be manifold and range from abiotic factors such as changes in temperature, or physiological factors such as nutritional or health state, to more complex causes such as directional or stabilizing selection via mate preferences, resulting in more dynamic or more static signal traits (Gerhardt, 1991; Eberhard and Treschnak, 2018). We explored causes of variability in male vibratory communication signals of male *K. biedouwense*, focusing first on the abiotic factor temperature. Temperature shifts can crucially affect communication signals in poikilothermic animals such as arthropods, and the communication system has to cope with this problem (e.g. Heller, 1986; Ronacher et al., 2004). We hypothesized that especially the temporal parameters of the vibratory signals (e.g. pulse repetition time, pulse train repetition time, and pulse train duration) will be affected by temperature. Additionally, we investigated if male age and body condition, expressed as size and weight, influence the production of vibratory communication signals. Females in various animal species have been found to preferably mate with males of a particular age (Brooks and Kemp, 2001), either because older males are better at acquiring resources important for female reproduction or outcompete younger rivals (e.g. Clutton-Brock and Albon, 1979; Rasmussen et al., 2007), or because females actively select males of a certain age, based on characteristics of male mating signals. Many examples exist that found male signals to vary with age, such as coloration and mating display (Miller and Brooks, 2005), acoustic (Hoikkala et al., 2007) and vibratory mate attraction signals (De Luca and Cocroft, 2009; Lubanga et al., 2016; Conrad et al., 2017). Considering female choice as an important factor for the evolution of substrate vibrational signals in males, we hypothesized that some characteristics of the male signals vary with body condition and age, while other characteristics might be more stable and used in species recognition or mate choice (due to stabilizing selection; see e.g. Gerhardt (1991).

Species recognition not only relies on the sender of a species-specific mate attraction signal, but also on the ability of the receiver to consider the sender an appropriate conspecific mate. It is therefore crucial to know the decision cues receivers use for signal recognition, as it may allow insights into the mechanisms and evolution of the neuronal circuitry underlying signal recognition, and into the coevolution of song pattern and recognition during speciation (Schul, 1998; Bailey et al., 2017). In some orthopteran species, which communicate via airborne sound signals, it has been shown that closely related species exhibit recognition patterns quite different from each other: While one species may rely on stable duty cycles of a song pattern (i.e. ratio between signal duration and period), others recognize pause lengths or pulse durations (von Helversen and von Helversen, 1994v; Schul, 1998). Thus, despite a homologous neuronal circuit for pattern analysis, different recognition algorithms evolved (Hennig et al., 2004, 2016; Bailey et al., 2017). Additionally, revealing signal recognition algorithms in related species will help to identify targets of sexual selection and constraints during evolution of pre-mating isolation and reinforcement (Coyne and Orr, 2004). We tested the decision cues in female *K. biedouwense* using one-choice experiments with various playbacks covering a broad range of temporal pattern variations within and beyond the conspecific male vibratory signals.

In this study we therefore investigate the signal recognition algorithm used by *K. biedouwense*, show that temperature coupling seems important in this species, and identify vibratory signal traits presumably targeted by selection during evolutionary divergence.

2. Material & methods

2.1. Animals

K. biedouwense males and females were collected as nymphs near Clanwilliam, Western Cape Province, South Africa (32.212 °S, 18.868 °E) and additional females at Biedouw Valley, Western Cape Province, South Africa (32.141 °S, 19.265 °E) in August - September 2016 and 2017. Individuals were transferred to the laboratory of Dr. Mike D. Picker, Zoology Department, University of Cape Town and kept separately in small plastic containers (height 50–90 mm, diameter 55–95 mm) at room temperature (approx. 21 °C). Males and females

were kept in separate rooms and reared to adulthood on *Drosophila* sp., termite nymphs (*Trinervitermes* sp.), and small crickets which were provided at least every second day. Water was supplied ad libitum by a saturated piece of paper towel. To test the impact of temperature and body condition on male vibratory signals, 20 adult males were used in 2016. In 2017, 10 males were used to analyze the effect of age on male vibratory communication signals. To assess the natural temperature ranges that adult *K. biedouwense* usually experience over a day, we measured temperature and humidity across seven consecutive days at the locality where the insects had been collected (Clanwillam Dam), using three data loggers fixed at different positions within a bush (Kestrel 4000, KestrelMeters, PA USA; 2x EasyLog EL-CC-1 Range, Lascar electronics, UK).

For the female response experiments, we used 31 females collected at Clanwilliam and Biedouw Valley in 2017 (seven females from Biedouw Valley and 24 females from Clanwilliam); for all experiments, adult females were used 7–18 days after the final molt.

2.2. Male vibratory signals

2.2.1. Testing the effect of temperature

After the final molt, 20 males were placed in a climate chamber set to 15 °C and left there 24 h for acclimation before recordings were conducted. For recording male vibratory signals, a single individual was placed on the membrane of a 5-inch loudspeaker (NWX-50358SQ, low-midrange loudspeaker 80–20,000 Hz, impedance 8 Ω, Nippon America Co., Nagoya, Japan). Animals were motivated to tap by imitating a female vibrational call with a pen tapped on the edge of the loudspeaker. If a male did not respond within a window of five minutes, it was removed and the next individual was tested. For recordings we used a Laser-Doppler Vibrometer (PDV-100, Polytec GmbH, Waldbronn, Germany), set to a velocity measurement range of 20 mm/s, with the laser directed perpendicular to the center of the loudspeaker membrane. Recordings were digitized (USB-connection-box VIB-E-220, Polytec, Waldbronn, Germany) and stored using the Software VibSoft 5.2 (Polytec GmbH, Waldbronn, Germany). Recordings were conducted always at the same time of the day (between 11 am and 3 pm). After the recording session in one temperature regime was finished, the temperature was increased by 5 °C and the males left there 24 h for acclimation until recording started again. Temperature was only increased from 15° to 30 °C in 5 °C steps due to technical reasons concerning the climate chamber. Males were provided with prey and water ad libitum throughout the experiment.

Subsequent to the experiment, all recorded males were weighed using a micro-scale and then anaesthetized by chilling and fixed in 70% ethanol. Pronotum lengths were measured as a proxy for total body size under a Zeiss Discovery V.20 MRc Stereomicroscope with 0.63x Objective, connected to a Zeiss AxioCam MRc, using the corresponding software AxioVision 4.8 (all Carl Zeiss AG, Oberkochen, Germany). The residuals of a linear regression of size and weight (both ln-transformed) were defined as a condition index for each male (Jakob et al., 1996). Thus, males with a positive residual value had a better body condition than average, while males with negative residuals were too light in respect to their size, thus had a worse condition than average.

2.2.2. Testing the effect of age

Adult male heelwalkers started emitting vibratory mate attraction signals ca. 3–4 days after the final molt. Subsequently, ten males were recorded as described above, every 1–3 days for a period of 25 days in a silent laboratory room with a mixture of artificial and natural light at room temperature (22 ± 1.4 °C). The first two recording sessions (day 1 and 5) were conducted with the loudspeaker only, thus, amplitude measurements could not be done here. All other recordings were performed with the laser vibrometer. Recordings were conducted always at the same time of the day (between 2 pm and 5 pm) and all males were provided with prey (*Drosophila* flies) and water ad libitum throughout the experiment. Unfortunately, weighing was not conducted after each recording in 2017; size measurement was done as above, taking pronotum lengths as proxy for overall body size.

2.2.3. Data analyses

We analyzed all vibratory recordings using VibSoft 5.2 or 5.3, measuring all parameters as set by Eberhard and Picker (2008): pulse repetition time, pulse train (PT) duration, number of pulses per PT, PT repetition time (PTRT), and inter-PT-interval (IPI = pause; Fig. 1a). Additionally, we measured the maximum amplitude (in mm/s) for each PT. To minimize observer bias, blinded methods were used when all behavioral data were analyzed. For each male, we calculated a median value for each parameter and treatment. For the temperature experiment, we calculated mean Q10 values using the 20 °C and 25 °C treatments only, since at 15 °C and 30 °C only a few males responded. The Q10 temperature coefficient represents a measure of the rate of change of a biological or chemical system as a consequence on increasing the temperature by 10 degrees (Robertson and Money, 2012; Eberhard et al., 2014). We used Wilcoxon signed rank tests to determine if the obtained Q10 values differed from 1 (= no change with temperature).

To reduce the parameters for analysis of the temperature treatment, we first conducted a principal component analysis (PCA) with all measured signal parameters. This resulted in two principal components (PC) that were used as response variables in the models. To analyze the effect of temperature on the PCs we used linear mixed models with the PC as response and temperature treatment and condition index as fixed factors. Male ID was included as random factor to account for the repeated measures. A null model without fixed effects was also created to be compared to the model with effects. For the age experimental group, the signal parameters were used directly in linear mixed models with random effects (male ID). Since the recordings differed slightly in temperature at the time of recording, we corrected all parameters using the calculated Q10-coefficients to standardize all values to 20 °C recording temperature. We included the day of recording (day 1 = onset of vibratory signaling, ca. 3–4 days after the final molt) and pronotum length (as proxy for body size) in maximum models and then excluded body size if there was no significant effect to reduce model complexity. Model quality (normality, homoscedasticity) was visually assessed and the influence of the given factors on the response variables was calculated using marginal and conditional R^2 values. All analyses were done with R (Version 3.3.3, The R Foundation for Statistical Computing, https://www.r-project.org/) using the packages: ggplot2, car, lme4 and MuMIn, or with SPSS (IBM SPSS Statistics, Version 22, IBM Corporation). Figure plates were arranged with CorelDRAW (X7, Version 17.5, Corel Corporation).

To compare age-related variation in signal parameters within males to the variation among males, we calculated coefficients of variation (CV = (SD/mean) *100). We first determined the CV for each male over all recording days to calculate a mean CV for within-male variability in vibratory signaling parameters due to age. Additionally, we calculated among-male CVs for each recording day and then across all recordings to obtain a measure of among-male variability in the communication traits.

2.3. Female choice

2.3.1. Experimental setup

Behavioral tests were conducted in an environmental room at constant temperature of 24.5 ± 0.5 °C and approximately 43.5 ± 4% RH. The insects were placed singly on a wooden stick (4–6 mm diameter, 60 cm length), which was collected at the field site in Clanwillam. The stick was fixed in a pot of sand, standing on a 10 cm thick folded cloth to damp vibrations coming from the ground. Playbacks were provided via a loudspeaker (5", Visaton SC 13, broadband speaker, impedance 8 Ohm, Visaton GmbH & Co. KG, Haan, Germany), communicating with the setup by a thin wooden stick (5 cm long, 2 mm in diameter) fixed

perpendicularly to the middle of the loudspeaker membrane by double-sided sticky tape and glue pads. Vibratory stimuli were applied to the setup via this rod, which was fixed on the stem, 35 cm above the sand. The loudspeaker was directly connected to the audio-output of a PC. Stimuli were played via Audacity (Version 2.1.1, http://audacityteam.org). Vibrational responses of females on the setup were monitored through a Laser-Doppler Vibrometer (PDV100, Polytec, Waldbronn, Germany) set at a velocity measurement range of 20 mm/s. Vibrations measured by the PDV100 were digitized (USB-connection-box VIB-E-220, Polctec, Waldbronn, Germany) and stored on a PC using the software VibSoft 5.3 (Polytec, Waldbronn, Germany). The laser beam was positioned perpendicular on the wooden stick, 4 cm above the sand ground, which was isolated with aluminum foil in this area.

The amplitude of the playback signals was approximated to the signal naturally emitted by a drumming male (= 2.2–3.2 mm/s). For this, preliminary tests had a *K. biedouwense* male drumming on the setup at the location of the playback loudspeaker. The volume of the playbacks was adjusted manually before the start of the main experiments by regulating the computer output volume to match the signal amplitude of the drumming male (2.6 mm/s). Measuring the played back signal on seven different locations on the wooden stick (0, 6, 11, 16, 21, 26, 31 cm distance from the vibration source) confirmed that the temporal pattern of the playbacks did not change when transmitted through the stick. Velocity amplitudes did vary a bit throughout the setup (2.6 mm/s close to initial measuring point and 4 mm/s close to the vibration source), but this was considered natural since we had calibrated the playbacks to a male drumming at the position of the playback source.

2.3.2. Stimulus generation

To assess female response arrays, artificial vibrational signals were generated using CoolEditPro software (Version 2.1, Syntrillium Software Corporation, USA) and played back to a female sitting on the experimental setup. A vibratory pulse was defined as one sine wave of 4 ms length, falling and rising once to a maximum amplitude (Fig. 1b–d). A model of the mean conspecific male song, based on mean values of recorded male calls from previous years (Eberhard and Picker, 2008; Eberhard and Eberhard, 2013; Eberhard and Treschnak, 2018) served as a control. All pulses within the generated pulse trains had the same amplitude (Table 1, Fig. 1b–d). For the test-playbacks, the temporal characteristics of the vibrational signals were varied in pulse repetition time, PT duration and number of pulses per PT. Altogether, 20 different playback files were designed (see Table 1, Fig. 2 for details). Each playback file consisted of a one-minute stimulus and 2 min of silence, resulting in 3 min duration per test. Within each 1 min stimulus, PTs were repeated between 5 and 50 times, depending on PT and pause lengths (Table 1).

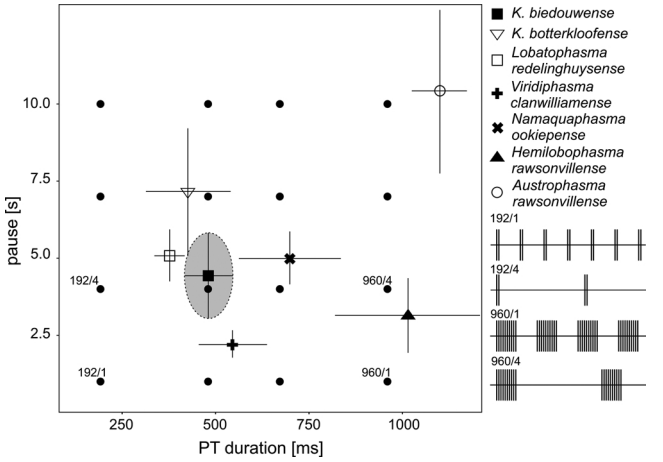

Fig. 2. Patterns of pulse train / pause durations of 16 playbacks with stable pulse repetition time of 96 ms used for behavioral tests. Black dots show the test pattern combinations of PT durations and pauses used for playbacks. Other symbols depict the mean pattern combinations of different heelwalker species (irrespective of the pulse repetition times). Lines depict standard deviations for the mean values. Grey area shows the mean value ± sd for *Karoophasma biedouwense* male vibratory signals recorded at 20 °C. Some example playbacks are shown on the lower right, numbers label the playback-IDs in the graph.

2.3.3. Experimental procedure

For each experiment, a female was placed on the setup, close to the laser beam of the recording laser vibrometer (ca. 6 cm above the sand ground). After 2 min of acclimation, the control signal was played via the computer and loudspeaker through the rod onto the setup. If the female did not answer by tapping towards this control playback within three minutes, it was put back to its cage and used again on another day. If the female reacted by tapping her abdomen on the stick, the

Table 1
Control and test signals that were played back to each female during one choice behavioral tests. The control was designed using mean values of male calls recorded in previous studies (Eberhard and Picker, 2008; Eberhard and Eberhard, 2013); 16 test signals varied the coarse temporal structure of the calls while keeping the fine structure (pulse repetition time) constant. Three playbacks had stable PT durations and repetition times while varying the number of pulses per PT, thus varying pulse repetition times. PRT – pulse repetition time; PT – pulse train.

Test-ID	PRT [ms]	Number of pulses per PT	PT duration [ms]	PT repetition time [s]	Pause [s]	Duty cycle	Total PTs in playback
Control	96	5	480	4.70	4.22	0.10	12
192/1	96	2	192	1.19	1	0.16	50
192/4	96	2	192	4.19	4	0.05	14
192/7	96	2	192	7.19	7	0.03	8
192/10	96	2	192	10.19	10	0.02	5
480/1	96	5	480	1.48	1	0.32	40
480/4	96	5	480	4.48	4	0.11	13
480/7	96	5	480	7.48	7	0.06	8
480/10	96	5	480	10.48	10	0.05	5
672/1	96	7	672	1.67	1	0.40	35
672/4	96	7	672	4.67	4	0.14	12
672/7	96	7	672	7.67	7	0.09	7
672/10	96	7	672	10.67	10	0.06	5
960/1	96	10	960	1.96	1	0.49	30
960/4	96	10	960	4.96	4	0.19	12
960/7	96	10	960	7.96	7	0.12	7
960/10	96	10	960	10.96	10	0.09	5
PRT240	240	2	480	4.70	4.22	0.10	12
PRT69	68.6	7	480	4.70	4.22	0.10	12
PRT48	48	10	480	4.70	4.22	0.10	12

other 19 playback signals were played back to her in a randomized order. After all playbacks had been played, the control playback was used once again to test for the female's motivation to still answer to an 'attractive' male signal. If the female did not answer to this control, we omitted all tested playbacks that had not been answered until the one that had been answered last. During all playback tests, we noted absence/presence of calling, length of tapping intervals during the three minute test trial, latency of answer after playback onset, and searching behavior including movement, movement latency, and distance moved. The number of emitted pulses was determined based on the recordings of the laser-vibrometer and the protocol. Each female was tested once with all 20 playback signals. After the experiment had finished, females were weighed and then chilled in a fridge and fixed in 70% ethanol for biometric measurements. All experiments were conducted during the day (between 10 am and 7 pm), within 9 days (11. – 19.09.2017) to reduce any effects of age or feeding status.

2.3.4. Data analysis

Since females have never been observed to tap without an appropriate stimulus (Eberhard and Picker, 2008), we considered no tapping as response towards a non-attractive/not recognized playback signal. Therefore, we first tested the absence/presence of tapping for all playbacks, using Cochran's-Q-Test and Wilcoxon's signed-rank posthoc tests. Subsequently, for each female that did tap in response to a playback, we calculated a relative response by dividing the number of pulses emitted during the test by the pulses produced during the control stimulus. This was necessary since we observed high variability in emitted pulse numbers between individual females. The relative responses were then used to build the response array graphs, while absolute tapping numbers were used to test for individual differences in responses in comparison to the pulses emitted towards the control (Wilcoxon signed-rank tests against the control, Bonferroni corrected). Additionally, we tested for differences in latency times, since more attractive calls would be answered sooner than less attractive ones. In some trials, females tapped immediately prior to the start of a playback, thus, a correct latency time could not be determined in those cases.

The datasets generated and analyzed during this study are available from the corresponding author on request.

3. Results

3.1. Male vibratory signals: effect of temperature

One week of continuously recording temperature and humidity every ten minutes from 15. – 22. August 2017 at Clanwilliam Dam revealed that *K. biedouwense* experiences temperatures between 2.3 and 34 °C (mean: 14.8 ± 6.9 °C), and 12 to 100% (mean: 52 ± 27.0%) relative humidity throughout a day at this site. Mantophasmatodea are thought to be most active during dawn and night; between 6 and 10 pm temperature ranged between 9.5 and 27.5 °C (mean: 14.7 ± 4.0 °C).

In the 15 °C and 30 °C treatment, only a few of the male heelwalkers started to call in response to the given stimulus (30% and 35%, respectively). Therefore, we were only able to statistically compare the 20° with the 25 °C treatment, since most of the males (80–95%) exhibited calling behavior. Changes in temperature had a significant effect on almost all measured signal parameters (Fig. 3a-f). While the temporal parameters decreased with increasing temperature with a Q10 of around 0.65, the number of pulses per PT as well as amplitude increased with heating from 20 to 25 °C with Q10 values of 1.3 – 1.5. However, the maximum velocity of pulses decreased again at 30 °C (Fig. 3d). The duty cycle also increased slightly for most males between 20 °C and 25 °C (see Fig. 3e-f; mean Q10 = 1.60) but remained quite stable over all four temperature treatments (Fig. 3e; 20–30 °C: Friedman ANOVA, $Q = 5.33$, $p = 0.07$, $N = 6$). The PCA of all measured vibratory signal parameters returned two PCs with Eigenvalues > 1, explaining 77.22% of the variability in the data. The first PC was closely associated with pulse repetition time, PT duration, PTRT, and IPI (pause). The second PC depended on the numbers of pulses per PT, PT duration, and duty cycle (Supplementary table S1). The linear mixed effect models including the factors temperature treatment and male condition showed that PC1 and PC2 were both significantly affected by temperature (Table 2, Fig. 3g,h): PC1, which represented both the temporal coarse signal pattern and fine scale pulse repetition times, decreased continuously with increasing temperature and was thus significantly affected by temperature, but not by the condition of the males (Fig. 3i). PC2, which was best associated with the number of pulses per PT and duty cycle, was less affected by temperature but varied more with male condition, since males in better body condition produced more pulses per PT and higher duty cycles, irrespective of ambient temperature (Fig. 3j).

3.2. Male vibratory signals: effect of age

Age significantly affected the individual males in most of the measured signaling parameters (Table 3): while pulse repetition time within a PT slightly increased (Fig. 4a), and the number of pulses within and duration of a PT slightly decreased over time, the drumming amplitude considerably decreased with male age (Fig. 4b). Only PT repetition time did not change over the 25 day recording period and remained overall stable (Table 3). Thus, duty cycle decreased as well with increasing age (Fig. 4c), due to the shorter PT durations. In the available dataset we found duty cycle significantly correlated with body size, since larger males exhibited overall higher duty cycles, irrespective of the day of recording (Table 3, Fig. 4d). Except for duty cycle and signal amplitude, marginal R^2 values (i.e. those associated with only the fixed effects 'age' and 'body size', if included in the models) were very low, so age had actually only little (though significant) effect on the given parameters, while conditional R^2, which includes random effects (individual male ID) as well, was always higher (Table 3).

Coefficients of variation within- and between males ranged between 9 and 29% (Table 4), with the traits of shortest duration (pulse repetition time) having the smallest, and those of longer duration exhibiting larger variabilities. Among-male variation within and between recording days was very similar to within-male variability over age (Table 4).

3.3. Female choice

Females were tested in their answering behavior towards artificial vibratory signals. While 16 playback signals varied the coarse pattern of calls (PT durations and pause lengths) without changing the species-specific pulse repetition time (96 ms; Fig. 2), three playbacks contained the conspecific PT-pause pattern but varied the fine structure of the signals (pulse repetition times). First, we analyzed the absence/presence of a vibrational response towards a given playback. Females reacted differently towards the signals (Cochran-Q-test, $Q = 142.3$, $df = 19$, $p < 0.001$). They were selective towards pulse train durations since less than 50% of the females answered by tapping towards the shortest pulse train durations (192 ms, Fig. 5a). Females were less selective for pause duration, where the number of calling females did not differ from the control, as long as PT duration was not too short. However, there was also a tendency that fewer females reacted towards very short pauses of 1 s (Fig. 5a: 480/1, 672/1, 960/1). When only pulse repetition times varied (with stable PT duration / pause pattern), females reacted significantly less with tapping towards very long pulse repetition times of 240 ms, while they did not differ in their response probability towards shorter pulse repetition times of 69 ms and 48 ms (Fig. 5a). Note that response probability of females was significantly reduced towards all playbacks that exhibited only two pulses per PT (either those with short PT durations or long pulse repetition times – see Table 1).

If a female answered towards a playback, the number of emitted

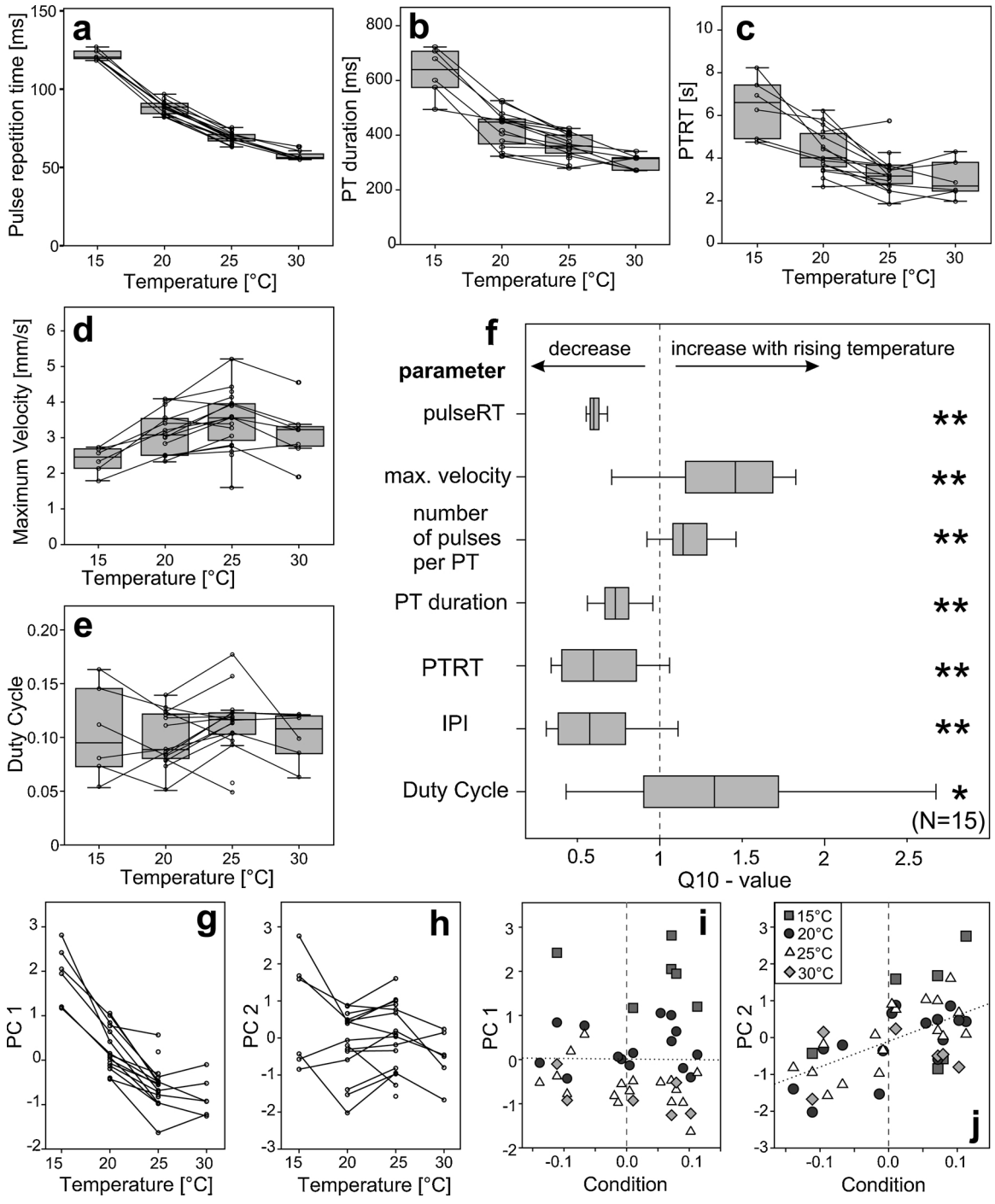

Fig. 3. Male vibrational signals and temperature: All temporal characteristics such as pulse repetition time (a), PT duration (b) and PTRT (c) decrease with rising temperature, whereas the maximally reached amplitude (d, measured as velocity) within each PT had a maximum at 25 °C. Duty cycles (e) of the signals did not change significantly over all temperatures tested. Lines connect the values of individual males over treatments. f) Mean Q10 values calculated for each male and parameter; here, only values from 20° and 25 °C were used. A Q10 value of 1 means no change of the given parameter, whereas Q10 < 1 represents a decrease of the parameter with increasing temperature. Stars depict significant difference of Q10 values from 1 (** $p < 0.01$, * $p < 0.05$). g–h) The two principal components that resulted from a PC of all signaling parameters measured plotted against temperature. PC1 (g), which is associated with the temporal structure of the male signal, changes significantly with temperature while there is no such clear trend for PC2 (h), which is best represented by duty cycle and number of pulses per PT. i–j) The two PCs plotted against male condition index: PC1 (i) does not correlate with condition, but PC2 (j) does (dotted lines are linear regression lines).

Table 2
Results of the linear mixed models with random effects for the temperature experiment with the principal components of male signaling parameters as response variables and temperature and condition index as fixed factors. Male individual ID was used as a random effect to account for the repeated measures. Significant effects are marked in bold.

Model coefficient	Fixed effect	Chi-squared	df	p	R2 marginal / R2 conditional
PC1	Temperature	216.25	3	< **0.001**	0.76 / 0.86
	Condition index	1.98	1	0.16	
PC2	Temperature	10.97	3	< **0.05**	0.45 / 0.73
	Condition index	13.79	1	< **0.001**	

Table 3
Results of the linear mixed models with random effects for the age experiment with male age (defined as the day of recording after onset of vibrational signaling; ca. 3–4 days after the final molt) and body size (pronotum length) as fixed effects and individual male ID as a random effect to account for repeated measurements. All signaling parameters were corrected for temperature using the Q10 values calculated from the temperature experiment. Only for duty cycle, body size had a significant effect and was used in the model; for all other parameters, this effect was not significant and therefore not used in the reduced models.

Model coefficient	Fixed effect	Chi-squared	df	p	R^2 marginal / R^2 conditional
Pulse repetition time	Age	5.41	1	< **0.05**	0.05 / 0.50
Number of pulses	Age	8.64	1	< **0.01**	0.08 / 0.52
PT duration	Age	19.81	1	< **0.001**	0.13 / 0.63
PTRT	Age	1.40	1	0.24	
Duty cycle	Age	14.27	1	< **0.001**	0.23 / 0.28
	Body size	4.14	1	< **0.05**	
Maximum amplitude	Age	21.79	1	< **0.001**	0.24 / 0.55

Table 4
Variation in vibrational signals over age expressed as coefficients of variation (CV). Average within-male CVs are similar to among-male CVs, indicating that the magnitude of age-related variation within males is comparable to levels of variation among males.

Parameter	Within male CV	Among male CV
Maximum amplitude	22.46	20.72
Pulse repetition time	8.68	9.15
Number of pulses per PT	19.35	19.25
PT duration	20.45	20.39
PTRT	25.27	26.62
IPI (pause)	27.12	28.50
Duty Cycle	29.16	29.36

pulses differed within females between tests (Friedman's test, Q = 35.7, N = 6, df = 19, p < 0.001; Fig. 5b). However, pairwise testing of emitted pulse numbers towards a playback and the control stimulus did not recover significant differences (Fig. 5b). Latency times from the start of a playback to initiation of tapping also differed between tests (Fig. 5c); females usually answered towards a playback within approximately 3.0 s, but latency times for playbacks 192/1 and 192/7 were much higher with 13.7 and 10.4 s, respectively. However, due to the low answering probability of females to those playbacks, a statistical analysis of these data was not possible.

For playbacks varying the fine structure of the male vibratory signals, tapping rate was affected differently (Friedman's test, Q = 8.07, N = 9, df = 3, p < 0.05): females exhibited higher tapping numbers towards 69 ms repetition times compared to shorter pulse repetition times (48 ms) and longer ones (240 ms). This was also true when using relative tapping rates in respect to the attractive playback signal (Fig. 5d). When looking at the various duty cycles of the signal patterns (caused by the various PT/pause combinations), females tapped less towards very short (< 0.05) and very long duty cycles (> 0.4) but apart

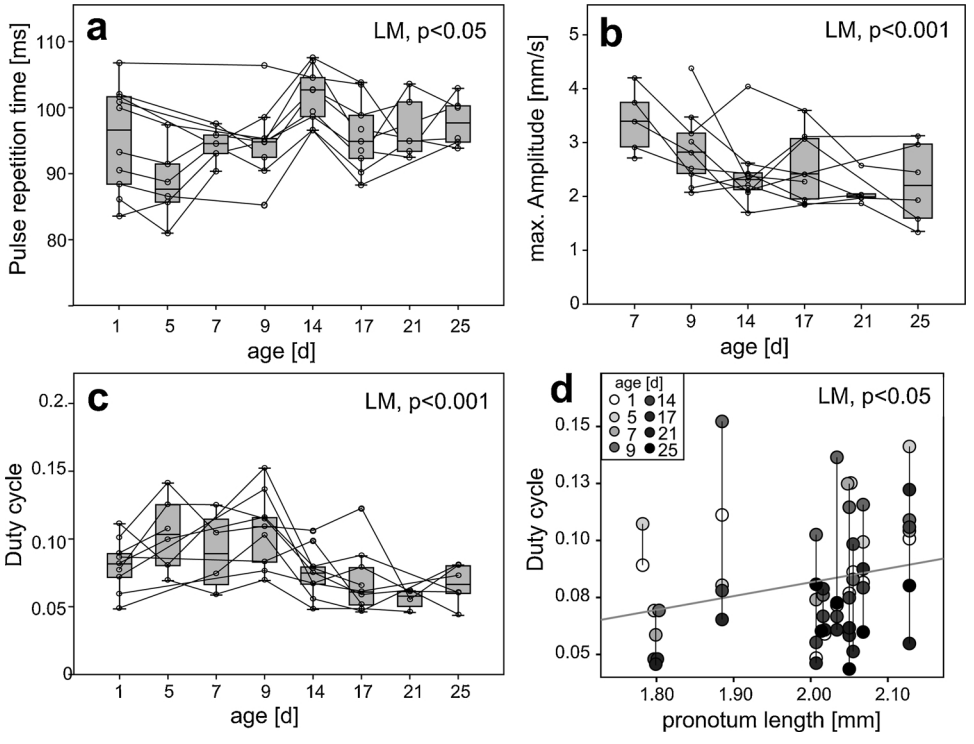

Fig. 4. Male vibratory signals and age: Pulse repetition time (a) changes slightly for each male over 25 days of age, while drumming amplitude (b) and PT duration (c) significantly decrease with age. Note that for vibratory amplitude, days 1 and 5 are missing due to recording procedure. d) Duty cycle correlates with body size (pronotum length), irrespective of the day of recording – circles connected with a solid line depict single individuals, and different grey values of circles represent different age groups. Note that for each age class, there is a positive relationship between body size and duty cycle (dotted line). P-values resulted from linear mixed models with random effects.

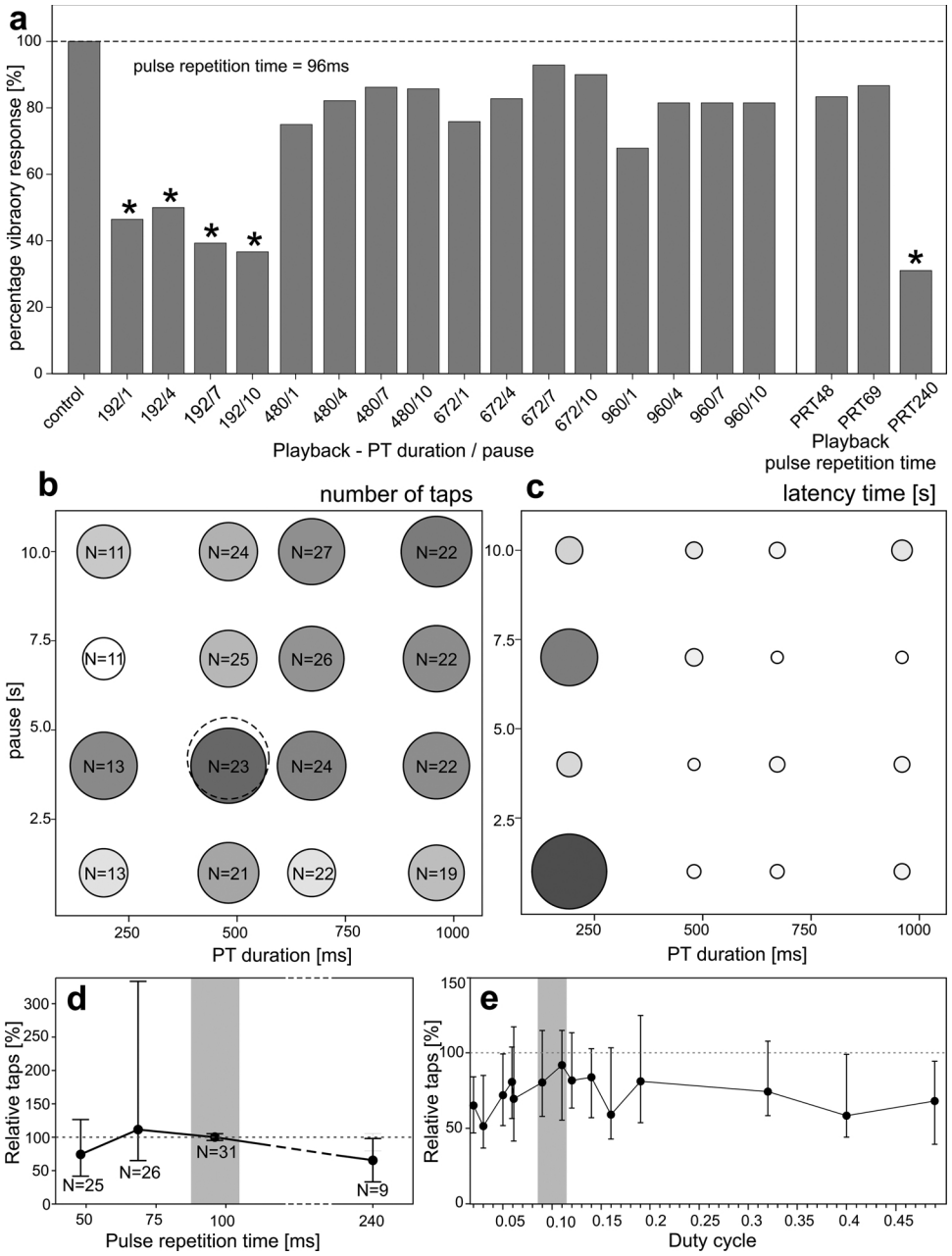

Fig. 5. Female preference for artificial playback signals: a) Percentage of females answering towards the control vibratory signal and the different playbacks. Stars depict significant differences in female answering probability in comparison to the control signal (Wilcoxon signed-rank tests, Bonferroni corrected, $p < 0.01$). Note that the control playback reached a response percentage of 100% by definition, since the tests only started when the female had answered towards this playback. b) Median relative taps emitted by females towards the various playbacks. The size and gray-values of the circles correspond to the relative tapping rate, the dashed circle depicts 100% tapping towards the control playback. For each playback, the number of answering females is given. c) Latency times between the start of a playback and the first tap of a female. Circles sizes indicate the time in s. d) Relative tapping rates of females during playbacks with varying pulse repetition times; dots show medians and bars depict 25–75 quartiles. A pulse repetition time of 96 ms was considered the species specific 'attractive' value, calculated from previously recorded males (gray area depicts mean male trait ± sd). Note that a shorter pulse repetition time of 69 ms was answered with relatively more vibratory pulses than the control playback. e) Relative tapping rates of females in respect to duty cycle (only PT/pause patterns with stable pulse repetition times shown); circles show medians and bars depict 25–75 quartiles. Gray bar shows the mean duty cycle ± sd (= 0.1 ± 0.015) produced by males of ca. 5–10 days of age).

from that, no obvious preference in terms of tapping rates could be revealed, mostly due to the low sample size compared to signal combinations and due to the high variability in female tapping rates (Fig. 5e).

4. Discussion

In this study we show that male vibrational signals exhibit intraspecific variability, both within and between individuals. Females showed some selectivity since they did not initiate vibratory signaling in response to a playback with only two pulses per PT, either because of short PT durations or very long pulse repetition times.

4.1. Temperature

Vibratory (incl. acoustic) signals can be characterized by their frequency content and pattern of amplitude modulations. The temporal pattern of these modulations is often crucial for signal recognition and preferences. Production of such signals depends on the movement of appendages and body parts like wings in crickets and bushcrickets, or hindlegs in grasshoppers, tymbals in cicadas etc. Since the speed of muscle contractions and the operation of intrinsic pattern generators depend on temperature (e.g. Janssen, 1992; Robertson and Money, 2012), the temporal pattern of amplitude modulations and calling patterns is, as a rule, strongly affected by temperature changes (e.g. Heller, 1986). Thus, parameters crucial for signal recognition, like pulse repetition rates or chirp periods, usually depend on temperature. This was also true for male vibrational signals in Mantophasmatodea, where all temporal parameters of the signals changed with increasing temperature (see Fig. 3).

Most tested males started calling in the 25 °C temperature treatment and also exhibited the highest signal amplitudes under this environmental temperature, while reactions were largely depressed at 15 and 30 °C (only a few males answered to the stimulation at all). We therefore hypothesize that *K. biedouwense* males prefer (or at least are most active at) a temperature around 25 °C. This does not match the mean

temperatures measured in the field (ca. 15 °C), but confirms the observation that heelwalkers hide in the cooler bushes or inside grass tussocks during hot days (when it gets > 30 °C) and only become active again at cooler temperatures around sunset.

Temperature effects are not confined to signal production but extend to the receiver part of the communication system. Temperature shifts affect properties of the receiver's sensory pathway, for example latencies, spike rates, the precision of spiking and, consequently, the upper limits of temporal resolution (Franz and Ronacher, 2002; Eberhard et al., 2014, 2015). An aggravated recognition problem arises in situations where the sender and the receiver differ in their body temperature (see e.g. Ronacher et al., 2004). Insects occur in various habitats characterized by large daily and/or seasonal temperature changes. Large temperature differences also occur at a microclimatic level, e.g. in a meadow where they may total up to 10 degrees between the ground and the top of vegetation (Römer, 2001). A communication system should be able to cope with the changes in the temporal pattern that appear in the temperature range the animals normally experience. Two different behavioral strategies were suggested to overcome this problem: (i) 'Temperature coupling' (Gerhardt, 1978) where the receiver prefers or even recognizes only signals from a sender calling at the same temperature as the receiver; (ii) evaluation of a ratio of two temperature dependent characteristics of the call that in itself is temperature independent (von Helversen, 1979v; von Helversen and von Helversen, 1994v; Ronacher et al., 2004). Examples where receiver's preferences and sender's calling rates change concordantly with temperature are known from crickets (Walker, 1957; Doherty, 1985; Hoy, 1992; Pires and Hoy, 1992a,b; Grace et al., 2004), grasshoppers (von Helversen, 1979v; von Helversen and von Helversen, 1981v; Skovmand and Pedersen, 1983; Bauer and von Helversen, 1987), planthoppers (DeVrijer, 1984), chironomids (Römer, 1970), spiders (Shimizu and Barth, 1996), and frogs (Gerhardt, 1978; Brenowitz et al., 1985). In the present study we show that temperature coupling could also be applied by Mantophasmatodea. In the case of the fine structure of the male vibratory signals, namely the pulse repetition times, females preferred signals produced by males calling at the same temperature as they experienced themselves. Female reaction in terms of emitted numbers of vibratory pulses was higher at a pulse repetition time of 69 ms compared to the control stimulus with pulse repetition times of 96 ms (Fig. 5d). The control stimulus resulted from a mean value calculated from male signals recorded at 20 °C, while the females were tested at temperatures of 24 °C (due to technical reasons, the climate chamber could not be set to a lower temperature). In this temperature regime, a mean male call would exhibit pulse repetition times of 78 ms (calculated from the temperature experiment). Concerning the coarser pattern of the male signals, females did not show such a selectiveness since only very short pulse trains and pauses were rejected by at least half of the tested females.

Analysis of the male signals recorded at different temperatures revealed that the duty cycle of the signals did not change markedly within individuals over all temperatures measured. Moreover, duty cycle, represented by principal component 2, was correlated with male body condition, irrespective of temperature (Fig. 3h,j). Females could therefore use this parameter as a temperature independent decision cue to answer or not answer a male signal, preferring larger duty cycles over smaller ones, as long as the fine structure of the calls – the pulse repetition time – exhibits the species specific duration. This is of course also true for the PT duration and number of pulses per PT, which are positively correlated with condition and might be used by females in mating decisions. However, in our behavioral experiments, females did not show specific preferences towards any particular duty cycles (Fig. 5e).

4.2. Age & condition

Intra-individual variability of male vibratory communication signals was induced by age: We found almost all measured parameters of male vibratory signals to vary at least slightly over 25 days. Coefficients of variation within- and between males ranged between 9 and 29% (Table 4), with the traits of shortest duration (pulse repetition time) having the smallest, and those of longer duration exhibiting larger variabilities. This corroborates the finding that variability of temporal patterns correlate with trait duration (Eberhard and Treschnak, 2018). The similar within-male and between-male CVs over age indicate that the magnitude of age-related variation within males is comparable to levels of variation among males (Table 4).

Adult heelwalkers were reported to live up to two months in the laboratory (Roth et al., 2014); however, in the field we did not find individuals after ca. 1.5 months after having discovered the first adults (Eberhard & Picker, personal observations). Most mating pairs are found within 1–2 weeks after the final molt, so we consider 25 days of recording male signals spans most of an adult male's reproductive phase. Male age did affect pulse repetition times, number of pulses per PT and PT duration, but the influence was very low (with very low marginal R^2 values) and we doubt that these small changes would induce a biologically meaningful change in female preference or impair their ability to recognize such signals. This is corroborated by the results of the female choice experiments, where females readily responded towards PT/pause combinations beyond the variation found with male age. In contrast, signal amplitude and duty cycle were more dramatically affected and might thus be a source for female preference. After 14 days of the onset of signaling activity of males, duty cycles decreased to around 0.06 (compared to 0.1 in younger males), a value where female reaction in terms of tapping rate dropped as well (Fig. 5e).

Interestingly, De Luca and Cocroft (2009) found similar changes with age in male Thornbug treehoppers, *Umbonia crassicornis*: both signal duration and amplitude significantly changed with male age. Variation in signaling parameters over time possibly reflect age-related trade-offs between different aspects of mate acquisition behavior. Younger males might be more at their peak physiological condition and are thus able to produce higher vibratory amplitudes than older ones. Decreasing amplitudes with age suggest a decline in condition or different investment into other aspects of mate acquisition such as mate searching or signaling effort. Such age-based changes in mate acquisition behavior have been found e.g. in butterflies (Fischer et al., 2008). Additionally, age-related changes in signal parameters could also be associated with changes in developmental processes that directly affect underlying neural and muscular systems controlling the production of signals (Elias et al., 2006). Since we do not have any data on whether heelwalker females would mate preferably with males of a certain age, we cannot conclude that females would choose older males over younger ones as found in other animals (Brooks and Kemp, 2001).

Younger males capable of producing higher drumming amplitudes and longer pulse trains would be able to transfer their signals further, thus having a higher probability to reach a receptive female. In playback trials using acoustic signals, females often prefer higher amplitudes over lower ones (Gerhardt and Huber, 2002), but this is less straightforward in substrate vibrational signals. In biotremology-based communication systems, signal amplitude doesn't seem to be a reliable trait for female choice (Cocroft and Rodríguez, 2005; Hebets et al., 2008; De Luca and Cocroft, 2009). Vibratory amplitude does not decrease monotonically with distance (Michelsen et al., 1982; Markl, 1983), especially along substrates as variable as dry, thorny bushes in which heelwalkers reside. Therefore we presume that females would not use absolute amplitudes to choose between males. Still, younger males may have an advantage, as their signals would simply reach further within a bush than those of older males.

Apart from intra-individual variability, we also find variation in signal traits between males of *K. biedouwense*, since at least some vibrational signal traits were correlated with body condition. This was true for PT duration, number of pulses per PT and especially duty cycle

for both the experimental temperature treatment group, as well as for the age group. In both groups of males, larger and heavier males were able to produce a higher number of pulses per PT, resulting in longer PT durations and higher duty cycles, irrespective of temperature or age (Figs. 3j, 4d).

4.3. Female choice

Many factors can influence male mating signals and female preferences. Females are thought to prefer a signal that is honest (meaning the accuracy with which the value of a male's trait indicates his overall health and condition), detectable within the environment, and which involves the least costs of preference (Schluter and Price, 1993). In Mantophasmatodea we found a strong correlation of some male vibratory mating signal traits with body condition and age, but no pronounced female preference for the coarse structure (PT duration and pause) of conspecific calls, despite the rejection of too short PT durations (or PTs with too few pulses). Similarly, treehopper *U. crassicornis* females did not prefer advertisement signals of older males; although, male signals varied with age and females were more often found to mate with older males (De Luca and Cocroft, 2009). The same was true in psyllids of the species *Aacanthocnema dobsoni*, where male vibratory signal traits correlated with age and body weight, but female responsiveness was not influenced by these differences (Lubanga et al., 2016). In this system, Lubanga et al. (2016) suggested that females respond to and mate with each male they encounter; they are polyandrous to avoid male harassment, and finally post- rather than precopulatory sexual selection mechanisms might be used by females to choose between males. Since female *K. biedouwense* answered with drumming towards a broad range of vibratory signal patterns, they might not be as selective towards vibratory signals as previously thought. This is in contrast to the results found in Eberhard and Picker (2008), who demonstrated that *K. biedouwense* females did not react at all by drumming towards the sympatric call of male *Viridiphasma clanwilliamense*. However, here also the fine structure of male calls, expressed in pulse repetition time, differed extensively between the two species. This signal parameter might therefore serve for species recognition where the two species occur together. Future experiments using real choices between different signals might reveal whether females use the information encoded in male vibratory signals for mate choice in direct comparisons, or in cryptic female choice by e.g. controlling copulation duration.

5. Conclusion

Here, we explored causes of intra- and inter-individual variability of male vibratory signals in heelwalkers. Temperature, as well as age, affected the temporal patterns of the signals, but not duty cycles. Duty cycle could therefore be used by females to select between males, since it was positively correlated with male body condition. However, our results show that females were not selective towards most PT / pause combinations used as playbacks. Substrate vibrational signals are crucial for Mantophasmatodea to reliably recognize and localize a mating partner within the complex vegetation they live (Eberhard and Picker, 2008; Eberhard and Eberhard, 2013). As soon as the searching male arrives at the stationary, tapping female, other communication modes such as chemical or tactile signals might be used by the female to assess the quality of the male. We hypothesize that male vibratory signals in Mantophasmatodea are thus used for species recognition and mate localization but are less important for precopulatory female choice.

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Compliance with ethical standards

All applicable international, national and/or institutional guidelines for the care and use of animals were followed.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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