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When virginity matters: age and mating status affect female responsiveness in crickets

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Behavioural assays in which animals recognize, localize or discriminate among signals are broadly useful for answering an array of biological questions, but results can be sensitive to the characteristics of individuals, including age and mating status. Researchers may prefer to use young, unmated individuals in experiments both to control for the effects of mating and because younger and unmated individuals may respond more often or more quickly. In natural contexts, virgin females are likely to behave differently than mated females, especially in animals that store sperm for future use, because the costs and benefits of mating are different for these two groups. In species in which multiple mating is common, individuals are much more likely to have mated at least once at the time of any given mating event than they are to be virgins, suggesting the use of virgin subjects in experiments is not reflective of most natural mating decisions. We conducted a literature review to sample the methods employed in empirical studies that use crickets in phonotaxis assays. Many studies draw conclusions based only on virgins, and methods vary widely with respect to the age of individual subjects. We then conducted an empirical study of the effect of mating status and age on female phonotaxis behaviour in *Teleogryllus oceanicus*, the Pacific field cricket. Our results show that both age and mating status affect some commonly used measures of female responsiveness in phonotaxis assays: older females begin moving more quickly in phonotaxis tests, while virgins have shorter response latencies. These findings are consistent with the idea that the cost of remaining unmated increases with age, and that virgin females benefit from an initial mating more than mated females benefit from additional matings.

Behavioural assays in which animals perform tasks related to recognition, localization or discrimination among signals are commonly used to investigate biological questions. In addition to measuring the strength and shape of selection on sexual signals (Blows, Brooks, & Kraft, 2003; Brooks et al., 2005; Tanner, Ward, Shaw, & Bee, 2017), these tasks are used to gauge the extent of reproductive isolation between divergent populations (Williams & Mendelson, 2010), explain the evolution of novel traits and trait loss (Basolo, 1990; Tinghitella & Zuk, 2009), make inferences related to the performance limits of animal sensory systems (Lee & Mason, 2017; Megela-Simmons, Moss, & Daniel, 1985), determine the neural and hormonal mechanisms of mate recognition and choice (Loher, Weber, & Huber, 1993; Ward & Blum, 2012; Ward et al., 2015) and assess whether animals recognize individuals (Feng et al., 2009; Tibbetts, 2002; Tumulty et al., 2018). Importantly, discrimination assays enable researchers to address a variety of questions using natural behaviours, that is, without training animals to respond to particular stimuli or testing environments. Despite expanding the range of questions we can answer, conditioning animals to respond to stimuli that do not naturally elicit behaviours can be time consuming and limit the number and diversity of biological systems investigated. The usefulness of discrimination assays is limited, however, by our understanding of the context in which biologically relevant decisions are made. Insufficient understanding of the biology of study organisms can affect our conclusions or ability to apply them more generally to other systems.

One of the most common contexts in which discrimination assays are used is in studies of mate choice, in which an individual is presented with one or more stimuli and responds by moving towards a signal or discriminating among several alternatives. To procure large samples and simplify statistical approaches, experimenters often either exclude females who do not respond in these assays, or set arbitrary cutoff limits for the length of trials. Those

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choices have practical value but may bias results because they primarily keep data from more responsive individuals, especially problematic for inferences made using two-stimulus tests, or assign an arbitrary maximum value to individuals that do not respond within the length of the test (‘right-censored data’; Kokko & Jennions, 2015; Stamps, Briffa, & Biro, 2012).

Many models exploring mechanisms of mate choice and its consequences are predicated on the assumption that females are mating for the first (and sometimes, only) time (Higashi, Takimoto, & Yamamura, 1999; Janetos, 1980; Real, 1990). In empirical studies, virgins are sometimes used in experiments explicitly because they are more likely to respond (e.g. Kodric-Brown, 1985). However, the costs and benefits of mating are not static across an individual's lifetime (reviewed in Jennions & Petrie, 1997). Life history traits such as age and mating status can have a pronounced effect on animal mating behaviour when individuals mate multiple times over the course of a season or a lifetime (Judge, Tran, & Gwynne, 2010; Lüpold, Manier, Ala-Honkola, Belote, & Pitnick, 2011; Prosser, Murray, & Cade, 1997; Wedell & Ritchie, 2004). For example, virgin live-bearing fishes are markedly more receptive than mated females, copulating with displaying males more quickly and more often than previously mated females (Liley, 1966). In wolf spiders, which have served as powerful models of multicomponent and multimodal signalling, males enjoy near-perfect mating success when courting virgin females, but mating success drops to roughly 50% when males court nonvirgins (Maklakov, Bilde, & Lubin, 2003). In insects, female receptivity decreases after mating in many taxa (Koudele, Stout, & Reichert, 1987; Leopold, Terranova, & Swilley, 1971; Ortigosa & Rowe, 2003).

Storing sperm for later use is common in animals that exhibit internal fertilization, including insects (Birkhead, 1993; Constantz, 1984; Orr & Brennan, 2015; Orr & Zuk, 2014; Simmons, 2001). Laboratory-based experiments in animals that store sperm often control for mating history, and potential effects of previous matings, by using exclusively virgin females as experimental subjects. This methodological choice can have consequences for the interpretation of results because past mating, or its lack, can influence current mating decisions. However, exclusive use of virgin individuals is unlikely to reflect the natural context in which sexual signalling takes place because age and mating status are necessarily correlated: individuals only get older and acquire more mating experience. An obvious consequence is that, in systems in which individuals mate multiply, individuals are much more likely to be mated than they are to be virgin at the time of any particular mating decision. It is therefore necessary to measure behavioural responses in virgin and mated individuals in the same species in order to quantify the extent to which laboratory methods under- or overestimate effects in natural populations.

We sampled the scientific literature to determine the range of variation in methodology employed in studies using phonotaxis assays in field crickets (Gryllidae), which are commonly used as models to understand sexual signal recognition, localization and discrimination. Our intention was not to present a systematic or comprehensive review, but rather to highlight (1) the breadth of the biological topics for which phonotaxis assays are used, (2) the frequency with which experimenters control for mating status by using virgin individuals and (3) the extent to which experimenters restrict and report the age range of their subjects. Field crickets are useful models in part because they use primarily acoustic signals to recognize, discriminate among and localize appropriate high-quality mates, and those signals are relatively easy to analyse and manipulate (Gerhardt & Huber, 2002; Huber, Moore, & Loher, 1989).

To assess the extent to which these methodological choices affect the broad applicability of results from phonotaxis assays for understanding natural signalling interactions, we conducted an experiment in *Teleogryllus oceanicus*, the Pacific field cricket, to quantify the effects of age and mating status on several commonly used measures of behavioural responsiveness in phonotaxis tests. Because unmated individuals suffer a fitness cost associated with being unable to produce viable offspring, we predicted that unmated individuals would be more responsive and therefore exhibit phonotaxis more often and more rapidly than mated individuals. Because the missed opportunity cost of remaining unmated becomes more significant with age, we expected that older individuals would be more responsive than younger individuals.

METHODS

Ethical Note

All experiments described here met the Animal Behavior Society/Association for the Study of Animal Behaviour guidelines for the ethical treatment of animals. We were given permission to import *T. oceanicus* to the mainland United States by the Department of Agriculture – Animal and Plant Health Inspection Service under permit number P526P-17-03395, which provided guidelines for the hand-collection of female crickets without the use of animal traps in Hawaii, the transportation of insect eggs to the mainland United States and the laboratory rearing of insects. Following behavioural testing, female crickets used in this study were returned to the laboratory colony.

Subjects and Animal Care

Subjects were 80 adult female *T. oceanicus* from a laboratory population descended from, and supplemented annually with, the fertilized eggs of wild-caught females from Hilo, Hawaii, U.S.A. The laboratory population size is maintained at a minimum size of 100 adult males and 100 adult females to minimize inbreeding. To control for maternal effects, we bred two generations of crickets in the laboratory prior to the experiment; that is, we used F2 offspring of the most recent field-caught females. To ensure virginity of experimental subjects and obtain adults of known ages, we isolated females from the laboratory population as late-stage juveniles and raised them to sexual maturity in individual enclosures. Juvenile subjects were checked daily for eclosion to adulthood. For each individual, the date of eclosion was designated ‘day 0’ and recorded. Each adult subject was then allowed to age until her randomly assigned testing day, which occurred between 6 and 14 days of age after eclosion. We chose six days as a minimum age because, while female field crickets are not phonotactic immediately upon adult eclosion, they usually become phonotactic prior to the sixth day after eclosion (Loher et al., 1993). To reflect the general experimental practice of limiting the age range of phonotaxis subjects, we chose an arbitrary cutoff age of 14 days after eclosion.

The laboratory population from which we drew experimental subjects was housed in 15-litre plastic containers in groups of about 30 individuals. Following isolation, experimental crickets were housed individually in transparent 188 ml plastic cups with lids. All crickets, including experimental subjects, were reared in insect growth chambers (Caron model 6025-2, Marietta, OH, U.S.A.) that maintain 26 °C, 75% relative humidity and a photoreversed 12:12 h light:dark cycle. All crickets were provided ad libitum access to water and Purina Rabbit Chow (Purina, Gray Summit, MO, U.S.A.), as well as egg cartons, or appropriately sized pieces of egg cartons, for shelter. Twice weekly, all crickets were transferred to clean containers and provisioned with fresh food, water and egg cartons.

Manipulating Mating Status

Females were assigned randomly to either the mated ($N = 40$) or virgin ($N = 40$) treatment. Females in the mated treatment were placed into a clean, empty cup with a randomly chosen adult male from the same population during the scotoperiod, when crickets are most active. The cup was then replaced in the growth chamber. After 1 h, the female was examined for the presence of a spermatophore to ensure that mating occurred. If no mating occurred, the female was returned to her cup and the mating exposure was repeated with the same male the following day. No female was mated to more than one male, and no male was assigned to more than one female subject. Of the 40 females in the mated treatment, approximately 30% did not mate at the first opportunity, but of these, all mated at the second opportunity. In the virgin treatment group, we performed a sham mating procedure, in which the male and female were introduced to the same cup for 1 h, but kept physically separated by a wire-mesh barrier. The barrier allowed the crickets to see, hear and antennate each other, but did not allow mating. Importantly, the barrier did not prevent male courtship behaviour, including the production of courtship song, or the transmission of chemical signals.

Song Model, Playback and Behavioural Assay

We constructed the acoustic stimulus after Simmons, Zuk, and Rotenberry (2001). We recorded *T. oceanicus* calling song in the field in Hilo, Hawaii at 24 °C and excised one pulse from each of the long chirp and short chirp segments of the song. We then concatenated the pulses together to generate a standard song model with a constant carrier frequency of 4810 Hz and population-specific temporal patterning based on means measured in the Hilo population from which our experimental females were descended (Fig. 1; Zuk, Rebar, & Scott, 2008). The song model was constructed to have a song period of 1.144 s and a duty cycle of 54%. Its proportion of long chirp, calculated by dividing the total duration of the long chirp portion by the song period, was 0.216. Within the long chirp portion of the song, pulses were 34 ms in duration with interpulse intervals of 17 ms. The interval that fell between the long chirp and short chirp was 63 ms in duration. Within the short chirp portion of the song, pulses were 29 ms long, with interpulse intervals within the chirp of 14 ms. Intervals between short chirps were 63 ms in duration. The intersong interval, between the final short chirp and the beginning of the following long chirp, was 77 ms.

We broadcast songs as uncompressed WAV files using SanDisk Clip Sport audio players (Western Digital Technologies, Milpitas, CA, U.S.A.) and Sony SRS-M30 Portable Computer Speakers (Sony U.S.A., New York, NY, U.S.A.). Playback of the song model was calibrated to a maximum sound pressure level of 80 dB (re. 20 μPa, fast RMS, C-weighted) at a distance of 1 m using an Extech 407750 Sound Level Meter (FLIR Commercial Systems, Inc., Nashua, NH, U.S.A.).

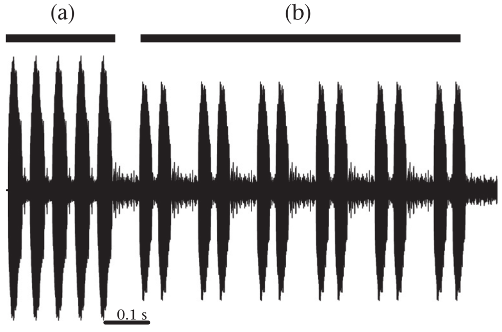

Figure 1. Waveform of the standard song model constructed using excised pulses from the long chirp (a) and short chirp (b) segments of song recorded in Hilo, Hawaii, at 24 °C.

Behavioural assays were conducted during the hour following the conclusion of the mating (or sham-mating) procedure. We performed single-stimulus phonotaxis tests to determine the effect of mating status on female responsiveness. The base assumption of these phonotaxis tests is that making antennal contact with the speaker is a proxy for female mate choice, in which a singing male is represented by the calling song model. Phonotaxis tests were performed under red light at 24 ± 2 °C in a semi-anechoic room. The testing arena was rectangular (155 × 34 cm and 32 cm high) and constructed from cardboard. Speakers were placed on the floor of the semi-anechoic room at each end of the arena, adjacent to an acoustically transparent mesh cut-out in the testing arena. At the beginning of each trial, the subject was placed at a distance of 1 m from the playback speaker and restrained under a cup, where it was allowed to acclimate in silence for 2 min. Playback of the acoustic stimulus began concurrently with the release of the subject from the cup. Trials ended when the subject indicated a response by antennating the playback speaker or else at the end of 5 min. We measured female responsiveness using four variables: latency to first movement (time elapsed between release from the cup and first locomotion), probability of responding to playback by antennating the playback speaker, latency to respond (time elapsed between release from the cup and antennating the speaker), and, in trials in which the subject did not respond, the distance the subject settled from the playback speaker. To control for side bias, we randomized and balanced which end of the arena was used for playback. Within sets of phonotaxis trials performed on the same day, we carried out trials in random order with respect to age and mating status of subjects. We also haphazardly assigned which individuals were tested at which ages, to avoid the systematic bias that might be introduced if we had tested all of the youngest individuals first.

Statistical Analysis

Continuous measures of female responsiveness were non-normally distributed according to Shapiro–Wilk tests of normality (latency to first movement: $W = 0.592$, $P < 0.001$; latency to respond: $W = 0.935$, $P = 0.027$; distance settled: $W = 0.900$, $P = 0.002$). We therefore log-transformed these measures prior to analysis. We included female age, measured as the number of days elapsed after eclosion, as a covariate in all models.

We used four separate generalized linear models to test for the effect of mating status on the four measures of female responsiveness: probability of response, latency to first movement, latency to respond and distance settled. The models examining latency to respond and distance settled were fitted using subsets of the data: females that did respond by antennating the speaker ($N = 39$) and females that did not ($N = 41$), respectively. We first examined full models that included age, mating status, and their two-way interaction. There was no response variable for which the interaction of age and mating status was significant; accordingly, the interaction terms were dropped from the final models presented here.

Because we fitted multiple models, we controlled for the false discovery rate using the Benjamini–Hochberg procedure (Benjamini & Hochberg, 1995). Using this method, P values are ranked in order of significance and a critical quotient is calculated for each. All tests whose P values are smaller than the highest-ranked value that is still less than the critical value retain significance. Original P values may then be adjusted by either scaling them by the total number of hypothesis tests divided by that value's rank, or else giving them the value of the next highest-ranked raw P value, whichever is smaller. We present both raw and adjusted P values for phonotaxis results.

All statistical analyses were done in R v.3.4.0 (R Core Team, 2018).

RESULTS

Our review of the literature (Table 1) showed that researchers conduct studies using cricket phonotaxis assays using subjects that often range widely in age (measured in days after adult eclosion) within studies, with no consistent pattern of the ages of females used across studies. Female subjects were primarily virgins at the time of testing except when the study explicitly addressed the effect of mating status, or when the assay took place using wild females of unknown mating status in the field.

In our experimental assay, female age ($\beta = -0.1641$, $t = -2.64$, $P = 0.010$, $P_{adj} = 0.040$; Fig. 2), but not mating status ($\beta = 0.1796$, $t = 0.65$, $P = 0.52$, $P_{adj} = 0.784$; Fig. 3a), affected the latency to first movement in phonotaxis trials. Older females began moving sooner after being released at the beginning of the trial, irrespective of mating status. Of 80 subjects, only two (one each from the virgin and mated treatments) did not move at all during the 5 min trial; these were assigned latencies to move of 300 s.

Among the subset of 39 females that responded to playback by making antennal contact with the speaker, there was a significant effect of mating status on the latency to respond ($\beta = 0.4175$, $t = 2.71$, $P = 0.010$, $P_{adj} = 0.040$; Fig. 3b), with virgin females

Table 1

A sample of 24 studies conducted in crickets using phonotaxis assays

Category	Topic	Female age (days after eclosion)	Female mating experience	Species	Source
Acoustic communication	Song recognition and predator avoidance	0–30	Virgin	*Teleogryllus oceanicus*	Moiseff, Pollack, and Hoy, 1978
Behavioural plasticity	Effect of acoustic experience on female reproductive behaviour and fecundity	14–16	Virgin	*Teleogryllus oceanicus*	Lierheimer and Tinghitella, 2017
Behavioural plasticity	Effect of age and perceived male density on female choosiness	13, 19 or 25	Virgin	*Gryllus lineaticeps*	Atwell and Wagner, 2014
Behavioural plasticity	Age-related plasticity in selectivity	5–7 or 21–31	Virgin	*Acheta domesticus, Gryllus pennsylvanicus, G. veletis, G. bimaculatus*	Stout et al., 2010
Cognition	Comparative decision making in mate choice	7	Virgin	*Gryllus bimaculatus*	Gabel and Hennig, 2016
Life history	Resource acquisition and mate choice behaviour	10	Virgin	*Teleogryllus commodus*	Hunt, Brooks, and Jennions, 2005
Mechanisms of communication	Effect of mating on phonotaxis behaviour	3–32	Virgin and mated	*Gryllus bimaculatus*	Loher et al., 1993
Mechanisms of communication	Genetic basis of female preferences for song traits	≥14	Virgin	*Laupala kohalensis, L. paranigra*	Shaw, 2000
Phonotaxis methodology	Receiver preferences for temporal patterns in choice and no-choice phonotaxis paradigms	14–84	Virgin	*Gryllus bimaculatus*	Doherty, 1985
Receiver mechanisms	Courtship song recognition	14–21	Virgin	*Teleogryllus oceanicus*	Balakrishnan and Pollack, 1995
Receiver mechanisms	Recognition of temporal patterns	10–14	Virgin	*Teleogryllus commodus, T. oceanicus*	Hennig and Weber, 1997
Receiver mechanisms	Neural basis of sound localization	10–21	Virgin	*Gryllus bimaculatus*	Horseman and Huber, 1994
Receiver mechanisms	Neural basis of sound localization	14–28	Not given	*Gryllus bimaculatus*	Schildberger and Hörner, 1988
Sensory systems	Evolution of mating preferences	Not given	Virgin	*Gryllus firmus, G. longicercus*	Gray et al., 2016
Sensory systems	Mechanism of discriminating among acoustic signals	9	Virgin	*Gryllus bimaculatus*	Hennig, 2009
Sexual selection	Effect of mating status and age on mating behavior	10–12 or 20–22	Virgin and mated	*Gryllus pennsylvanicus*	Judge et al., 2010
Sexual selection	Effect of acoustic experience on female preferences	6	Virgin	*Teleogryllus oceanicus*	Bailey and Zuk, 2008
Sexual selection	Male size and sequential mating preferences	10–15	Virgin and mated	*Gryllus bimaculatus*	Bateman et al., 2001
Sexual selection	Multivariate selection on acoustic signals	Unknown	Unknown	*Teleogryllus commodus, T. oceanicus*	Bentsen, Hunt, Jennions, and Brooks, 2006
Sexual selection	Female choice and time cost, age, nutrition, body size and size-relative reproductive investment	10 or 20–21	Virgin	*Acheta domesticus*	Gray, 1999
Sexual selection	Female preferences for acoustic and olfactory signals	10–14	Virgin	*Teleogryllus oceanicus*	Simmons, Thomas, Simmons, and Zuk, 2013
Speciation and reproductive isolation	Population divergence in calling song discrimination	7–30	Virgin	*Gryllus fultoni, G. vernalis*	Jang and Gerhardt, 2006
Speciation and reproductive isolation	Discrimination between conspecific and heterospecific songs in crickets from a hybrid zone	7–28	Virgin	*Gryllus firmus, G. pennsylvanicus*	Doherty and Storz, 1992
Speciation and reproductive isolation	Geographical variation in male song and female preferences	10–15	Virgin	*Teleogryllus oceanicus*	Simmons et al., 2001

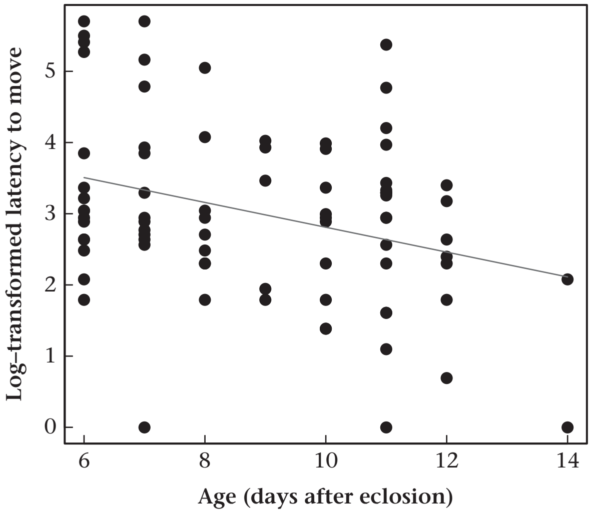

Figure 2. Effect of age, measured in days after eclosion, on the log-transformed latency to first movement.

approaching the speaker more quickly. The mean latency to respond for virgin females was 108 s, compared to 156 s for mated females. There was no significant effect of age on latency to respond ($\beta = 0.038$, $t = 1.06$, $P = 0.296$, $P_{adj} = 0.784$).

The probability of responding by antennating a speaker was 0.475 for mated females and 0.500 for virgin females. Mated females were somewhat, but not significantly, less likely to respond by antennating the speaker ($\beta = -0.0285$, $z = -0.27$, $P = 0.074$; Fig. 3c) than virgins. There was no effect of age ($\beta = -0.1330$, $z = -0.29$, $P = 0.784$, $P_{adj} = 0.784$) on the probability that a female responded to playback.

Among the subset of 41 females that did not respond to playback by antennating the speaker, we found no significant effect of mating status ($\beta = 0.1618$, $t = 0.31$, $P = 0.76$) or age ($\beta = -0.0671$, $t = -0.60$, $P = 0.55$) on the distance subjects settled from the playback speaker at the end of the trial.

DISCUSSION

The studies referenced in Table 1 reflect the breadth of methodologies employed by biologists using phonotaxis assays of crickets to answer a variety of questions. The ages of females used as subjects often spanned a fairly large range within studies and no particular convention was adopted across studies. Researchers primarily use virgin crickets in assays, except when they explicitly investigate the effect of mating on behaviour or use wild females with unknown histories. A common approach is to use virgin females of a relatively restricted age range. Here, we show that some measures of phonotactic responsiveness are sensitive to age as well as mating status in crickets. We suggest that while standardizing cricket behaviour by using only virgin females may control for the effects of mating history, results obtained in that way may not be representative of cricket behaviour in general. In addition, using a restricted age range, such as we have done here, does not completely prevent female age from influencing phonotaxis results. Thus, using all virgin females within a defined range of ages as phonotaxis subjects may fail to appropriately address population-level inquiries about mate choice and its consequences. It may be informative to replicate some designs across age groups or, at minimum, use female crickets of varying known ages and include age as a covariate in statistical models.

While both age and mating status affect some commonly used measures of responsiveness in single-stimulus phonotaxis tests, neither affected all response variables and there were no significant interactions. Similarly, Mautz and Sakaluk (2008) found that age and mating status both moderate selectivity in *Acheta domesticus*, but that these factors had different relative importance in pre- and postcopulatory sexual selection. Age has been shown to affect mate choice in insects, with older females generally being less selective (Ritchie, 1992; Ritchie, Butlin, & Hewitt, 1989; Watt, Carter, & Donohue, 1986). Older females in our experiment showed a shorter latency to first movement than younger females, which is consistent with the idea that the cost of remaining unmated and, conversely, the benefit of mating increase across an individual's lifetime. That age affected latency to first movement, but not latency to respond by antennating the speaker, may be a

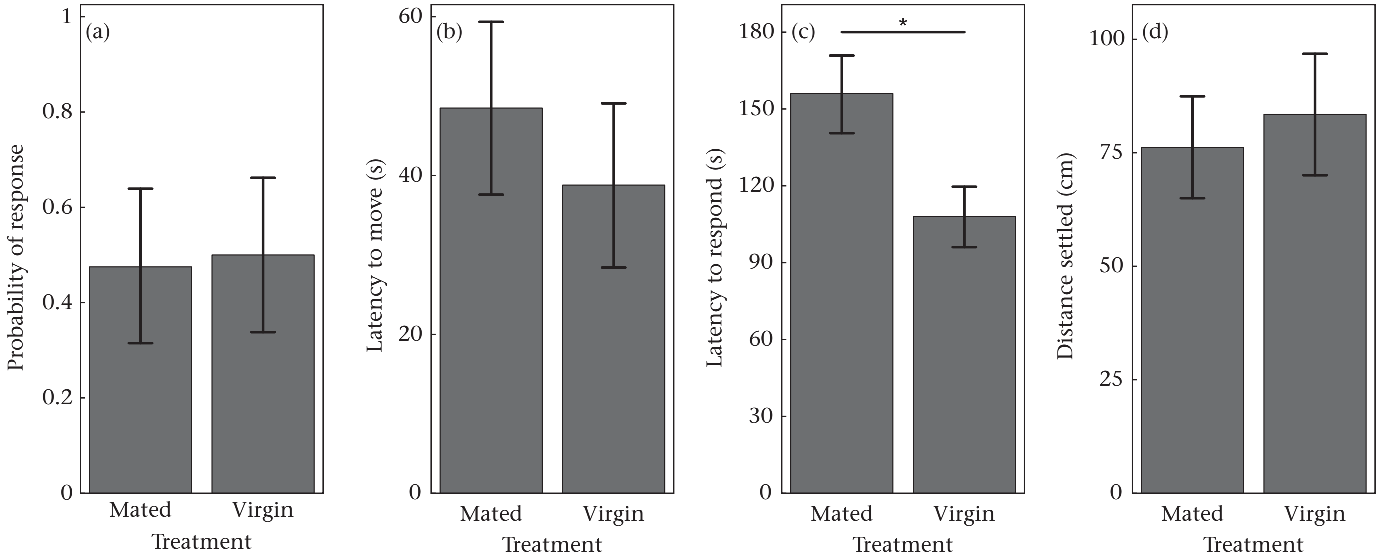

Figure 3. Measures of female responsiveness by mating status: (a) latency to begin moving in response to playback; (b) latency to respond by making antennal contact with the speaker; (c) the probability of responding by making antennal contact with a speaker in a phonotaxis test; and (d) for trials in which a female did not contact the speaker, the distance settled from the speaker. Error bars in (c) are 95% Clopper–Pearson (‘exact binomial’) confidence intervals; error bars in all other panels are standard errors of the mean.

consequence of different sample sizes (80 and 39, respectively) for those two models. Mirroring practices in the field generally, we tested females within a fairly restricted age range, beginning 6 days after eclosion when most females begin to be phonotactic, and ending at 14 days; despite this limitation, we nevertheless found that age affected one measure of female responsiveness. Field crickets are likely to live longer than 14 days past eclosion in the field (Zuk, 1987) and are routinely tested at older ages in laboratory experiments (Bateman, Gilson, & Ferguson, 2001; Doherty & Storz, 1992; Lierheimer & Tinghitella, 2017; Mautz & Sakaluk, 2008; Prosser et al., 1997). The effects of age on measures of female responsiveness may be even more pronounced when considering data collected using animal subjects that range in age over several weeks. As a practical consideration, using female subjects that vary in age may add considerable noise to data that rely on response latencies as primary response variables or to calculate phonotaxis scores, inflating the number of experimental subjects required and potentially biasing results.

The effect of mating status on female behaviour is likely to be most pronounced in species that mate multiply within a reproductive episode and store sperm for future use (Thornhill & Alcock, 1983). By contrast, in animals that mate singly within a reproductive episode or that exhibit external fertilization, the fitness consequences of a second mating may be independent from those of the first. Mated females are less responsive than virgin females in field crickets (Judge et al., 2010), and other animals that mate multiply and store sperm for future use, such as livebearing fishes (Pitcher, Neff, Rodd, & Rowe, 2003) and newts (Gabor & Halliday, 1997). In these systems, females are expected to be less responsive, more choosy, or both, after having mated, but they may mate multiply when they can ‘trade up’ in genetic quality by doing so (trade up hypothesis; Halliday, 1983; Jennions & Petrie, 1997, 2000; Pitcher et al., 2003). In our assay, virgin females responded more quickly to playback than mated females, which is consistent with the idea that virgins benefit from mating relatively indiscriminately, compared to previously mated individuals. However, in one sense, our experimental design limits inference about the effect of mating status because the behavioural assay was carried out immediately following the mating procedure. It may be that females become more likely to remate as a function of time elapsed since their last mating. If so, performing the behavioural assay immediately following the mating procedure may tend to overestimate the effect of mating on female responsiveness.

Latency to respond is a frequently used measure of female preference in behavioural assays in crickets and other animals, and the primary response variable for many single-stimulus behavioural assays (Wagner, 1998). The single-stimulus paradigm is employed in studies examining sexual selection, speciation or population divergence, response thresholds and to address other questions. Single-stimulus phonotaxis tests are advocated by some authors because, while two-stimulus choice tests provide information only about directional preferences, single-stimulus tests provide information about the absolute preferences that may persist even when the perceived encounter rate of potential mates is low (Dougherty & Shuker, 2014, 2015; MacLaren & Rowland, 2006; Wagner, 1998). When single-stimulus tests are used to present a series of different stimuli, an implicit assumption is that individuals have the same base motivation to respond across trials, but this may not be true when characteristics of individuals, such as age and mating status, are variable between subjects. Here, we show that in crickets, the outcomes of single-stimulus behavioural assays may be influenced by the age of subjects. Importantly, experiments assessing the phonotaxis behaviour of insects often use treadmill devices, in which an insect is tethered to a lightweight, rotating ball that can be used to measure its rate and direction of travel (Lee & Mason, 2017; Lierheimer & Tinghitella, 2017; Prosser et al., 1997). Our results in free-moving crickets are thus also applicable to experiments that measure walking speed using treadmill devices.

Virgins were no more likely to respond than mated females in single-stimulus phonotaxis tests. By contrast, in a study of *Gryllus pennsylvanicus*, Judge et al. (2010) showed that virgin females were more likely to copulate and had shorter latencies to mate than previously mated females. The effect of mating status may be variable among field cricket species or between assays that use phonotaxis as a proxy for mate choice and assays that measure mating behaviour directly. For example, when mating behaviour is measured directly, phonotaxis may be followed by production of male courtship behaviour, including courtship song, which could reinforce or counteract female behavioural differences observed in response to playback of calling (advertisement) song alone. Furthermore, we assayed female phonotactic response to a population standard signal and cannot, on the basis of these data, draw conclusions about whether mating status affects discrimination between two signals of differential attractiveness. Because the benefit of mating is greater for a virgin female than for a previously mated female, virgins may be less discriminating.

Our intention here was not to claim that phonotaxis results obtained using exclusively young, virgin crickets are not useful, but rather to point out that a detailed understanding of phonotaxis behaviours and their consequences under natural conditions necessitates taking characteristics of receivers, such as age and mating status, into account. We suggest that the inclusion of previously mated females in cricket phonotaxis experiments, contrary to common practice, may more accurately reflect natural behaviours based on signal recognition, localization and discrimination. Adequately accounting for the effects of age on phonotaxis behaviour may require the use of subjects that span the entire receptive period of phonotactic adults and the subsequent inclusion of age as a covariate in statistical models. Additional research is needed to closely examine how age and mating status affect discrimination among several alternative signals. Even within the field crickets, the number and diversity of species used to investigate questions related to signal recognition, localization and discrimination is not trivial; determining how characteristics of individuals, such as age and mating status, affect behaviour thus will require examination at the species or population level.

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