



Heterospecific courtship and sequential mate choice in sister species of field crickets

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During mate selection, females of most species encounter males sequentially and may use a variety of decision rules whereby the attractiveness of a current potential mate is affected by sampling of previous individuals. The perceived distribution of male quality might therefore affect female mate choice. Mate choice among closely related taxa may additionally be affected by a perceived rarity of conspecifics. Here we tested whether female mate choice in the field crickets *Gryllus rubens* and *G. texensis* is affected by prior experience with either conspecific or heterospecific males. Specifically, we tested whether female acceptance of conspecific or heterospecific males is influenced by prior exposure to a conspecific or heterospecific male. Our results indicate that there is a clear previous-male effect, but that it differs across species. When 'high-quality' (i.e. conspecific) males are common, females are predicted to prefer them. *Gryllus texensis* females matched this prediction by showing clear preferences for conspecific males after prior experience with them, but *G. rubens* females showed the opposite effect: they preferred heterospecific males after prior experience with conspecific males. When prior experience is manipulated such that 'low-quality' (i.e. heterospecific) males are encountered first, female choosiness should be relaxed. Females of both species fit this prediction and showed no preference for conspecific or heterospecific males after prior experience with a heterospecific male.

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It is well documented that female mate choice exerts strong selection on male secondary sexual characteristics (Andersson 1994; Kokko et al. 2006). One area of research into female mate choice has focused on how females assess the attractiveness of potential mates (Dombrovsky & Perrin 1994; Mazalov et al. 1996; Bateson & Healy 2005). A commonly used research approach is a simultaneous choice test, in which females are exposed to multiple males at once. The female is then able to assess the relative quality of all potential mates and select the most attractive mate. Although this approach is useful for species in which males display simultaneously and in close proximity to one another (e.g. many lek-mating species), it does not mimic the natural setting in species where males are spatially dispersed. In many species, females must actively search for males, and females of many taxa visit and assess multiple males before selecting a mate (Dale et al. 1990, 1992; Rintamaki et al. 1995; Backwell & Passmore 1996; Forsgren 1997). In such sequential

choice situations a female may use a threshold, template or 'best-of-*N*' rule to compare the males she encounters (Janetos 1980; Real 1990; Wiegmann et al. 1996; Wiegmann & Morris 2005). The dynamics of sequential female choice may be quite different than in a simultaneous choice situation, potentially leading to different outcomes (Bateson & Healy 2005; Ivy & Sakaluk 2007).

Experience plays a key role in mate choice decisions of females that search for males sequentially. The time spent searching for a suitable mate may be influenced by the costs and benefits of continuing the search, as well as the relative quality of the males encountered (Milinski & Bakker 1992; Gabor & Halliday 1997; Wagner et al. 2001). In sticklebacks, females become less choosy as the costs associated with the search become greater (Milinski & Bakker 1992). Similarly, female field crickets, *Gryllus integer*, face trade-offs between mate choice and predation risk, and are less choosy when risk is higher (Hedrick & Dill 1993). However, when search costs are low, females become choosier as the quality of previously encountered males increases: in newts and guppies, polyandrous females trade up in quality with each mate (Gabor & Halliday 1997; Pitcher et al. 2003). While it is clear that a 'previous-male effect' operates in many species (Bakker & Milinski 1991; Collins 1995; Gibson & Langen 1996; Bateman et al. 2001; Kavaliers et al. 2003), the implications of this effect are not always

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clear. Furthermore, females of some species use sequential search but appear to follow a fixed template without reference to previous mates (Ivy & Sakaluk 2007). The relative frequencies of 'previous-male effect' and 'fixed template' mate-sampling strategies are unknown, and further work in this area would be useful.

Sequential search combined with previous-male effects could have negative fitness effects for naïve females when the males they encounter are not conspecifics, but closely related heterospecifics. In closely related sister species, if sexual signals are not sufficiently divergent, previous experience with heterospecific males may greatly alter female sampling. How experience with heterospecifics influences mate choice decisions of females in sequentially sampling species has not been explicitly investigated.

Investigating sequential mate choice in sympatric sister species is additionally important as recent work has indicated that, under certain circumstances, females may actually prefer heterospecific males (Abt & Reyer 1993; Pfennig 1998). Such hybridization may be adaptive under certain circumstances, as it may free hybrids from developmental constraints of a parental genotype (Pfennig 2007), enable adaptive radiation of novel ecotypes (Arnold 1997; Seehausen 2004) and enable individuals of a rare species to gain fitness despite rarity of conspecifics. Conspecific rarity appears to be a widespread and perhaps general contributor to interspecific hybridization. Examples are found in fish, birds, mammals and molluscs (Hubbs 1955; Ribi & Porter 1995; Grant & Grant 1997; Ribi & Katoh 1998; Ribi & Oertli 2000; Thulin & Tegelstrom 2002). By examining patterns of sex chromosome introgression among 80 species pairs, Wirtz (1999) concluded that demographic rarity affects males and females differentially: females of rare species may readily mate with common heterospecific males (given a scarcity of conspecific males), but males of rare species are unlikely to mate successfully with common heterospecific females. This follows from the effects of sex-related differences in reproductive investment on choosiness: males of an abundant species may not discriminate against females of a rare species; however, we would expect females of an abundant species to have many potential conspecific mates, and so avoid mating with heterospecific males.

Field crickets (Orthoptera, Gryllidae, Gryllinae) are ideal for testing how previous experience with heterospecific males influences the mating decisions of females. Female field crickets listen to the calling songs of males and fly or walk towards the most attractive songs (Alexander 1961; Zuk & Simmons 1997). Although females are able to listen to and assess multiple males simultaneously (from the long-range calling songs of males), females visit males sequentially and are polyandrous (Sakaluk et al. 2002; Bretman et al. 2004; Bretman & Tregenza 2005; Ivy & Sakaluk 2005; Garcia-Gonzalez & Simmons 2007; Jennions et al. 2007). Females may also be intercepted by noncalling males (Cade 1979b, 1981; Zuk et al. 1995, 2006). Some species of *Gryllus* field crickets form viable hybrids in the laboratory (Harrison 1983; Smith & Cade 1987; Cade & Tyshenko 1990). Previous work with both *Gryllus lineaticeps* and *Teleogryllus oceanicus* has shown that female acoustic experience can modify subsequent female mating behaviour (Wagner et al. 2001; Bailey & Zuk 2008). Furthermore, female *G. integer* move among locations in a manner consistent with their recollection of the locations of previously sampled males (Wiegmann 1999). An additional advantage of field crickets as a study system for this question is that males are unable to force females to mate; copulation requires the female to cooperate by mounting the male, thus enabling spermatophore transfer (Zuk & Simmons 1997).

Gryllus rubens and *G. texensis* are recently diverged sister species that share a large zone of sympatry in the southeastern United States. Males are morphologically indistinguishable, and differ mainly in the pulse rate of their calling songs: 56 pulses/s at 25 °C

for *G. rubens* and 80 pulses/s at 25 °C for *G. texensis* (Gray & Cade 2000; Izzo & Gray 2004). Both species are currently abundant in nature with high temporal and spatial variations in density (Cade 1979a, 1989) and, in areas of sympatry, are likely to encounter one another frequently. Although male calling songs are species specific, males spend less time calling and more time wandering to intercept females when populations are at high density (Cade 1991; Cade & Cade 1992). Thus, there is an opportunity for females to encounter males without prior phonotaxis to calling song. Once a male and female come into contact, the male switches from calling song to courtship song. *Gryllus rubens* and *G. texensis* differ somewhat in their courtship songs; *G. texensis* has a faster courtship song than does *G. rubens* (Fitzpatrick & Gray 2001). In nonsequential no-choice mating trials, females of both species preferred conspecific courtship (Gray 2005). Recent molecular work has shown that *G. rubens* probably arose via peripatric speciation from within a subset of *G. texensis* (Gray et al. 2008). *Gryllus rubens* harbours very little genetic variation in mitochondrial DNA sequences compared to *G. texensis*; the haplotypes that *G. rubens* does have are mostly shared with *G. texensis*, and *G. rubens* (but not *G. texensis*) shows the molecular signatures of recent demographic and geographical expansion (Gray et al. 2008). Together these data suggest that *G. rubens* may have had a significant portion of its evolutionary history spent at small population size on the periphery of its abundant parent species.

Here, we tested how previous male experience influences female mate choice when prior males were heterospecific or conspecific. We predicted that females should use prior experience to gauge the availability of 'high-quality' (i.e. conspecific) males. When prior experience is with a conspecific male, females should be reluctant to trade down in quality and should prefer conspecific second males over heterospecific second males. When prior experience is with heterospecific males, females should relax their subsequent choosiness because high-quality mates are rare. Our basic methodology was to provide females of both species with males of both species sequentially but in random order. We predicted that the likelihood of female acceptance of a second male would depend on the species identities of both the first and the second male. Based on the inferred evolutionary histories of these species, we further predicted that female *G. rubens* would be more amenable to interspecific mating than would female *G. texensis*.

METHODS

Crickets

Although many *G. rubens* and *G. texensis* females can be distinguished using the relative length of the ovipositor (Gray et al. 2001), species identification of some females is problematic unless one rears offspring from each female separately and records the songs of each female's sons. To avoid possible ambiguity in species assignment, and to avoid having to rear each female's offspring separately, we collected females to initiate our laboratory cultures from allopatric populations; *G. rubens* were collected in Gainesville, Florida in 2002 and *G. texensis* were collected in Austin, Texas in 2002 and 2003. It may seem intuitive that studies examining reproductive isolation would use sympatric populations rather than allopatric ones; however, this is only true if one assumes a history of selection against hybridization in sympatry (i.e. reinforcement). Four previous studies explicitly examined potential differences both in calling song and in male and female courtship behaviour between allopatric and sympatric populations of these species, and all four studies found no difference between allopatric and sympatric populations of either species (Gray & Cade 2000; Izzo & Gray 2004; Gray 2005; M. Korody, N. Osborn &

D. A. Gray, unpublished data). Thus, we feel justified in our use of allopatric collections to initiate laboratory cultures.

Once laboratory cultures were established, crickets were maintained on a 14:10 h light:dark cycle at 25 °C. In the 'breeding bins' (121-litre trash cans), adults were provided Purina Brand dry cat food ad libitum, plastic water vials plugged with absorbent cotton, and a moist sand/vermiculite/potting soil mixture in which to lay eggs. Egg dishes were removed weekly, and placed into 51-litre 'nursery bins'. Hatchlings were raised in the nursery bins under identical conditions (25 °C, LD 14:10 h). As crickets neared the final moult, the nursery bins were checked daily, and all newly eclosed adults were removed and housed in individual 500 ml containers with food, water and an egg carton shelter. This isolation ensured adult virginity and allowed determination of sexual maturity prior to mating trials; adults of these species are sexually mature at 5–7 days post-adult moult. All cricket containers were kept in an environmental chamber so that temperature and light could be controlled. Prior to use in mating trials, males of both species produced calling songs, and females of both species were exposed to heterospecific and conspecific calling songs, which mimics natural calling song exposure in the field.

No permits were required for cricket collection, and all applicable legal and ethical guidelines for use of animals in research were adhered to in this study.

Mating Trials

Females were randomly assigned to participate in a pair of no-choice mating trials (i.e. one male with one female). All adults were virgins, 10 ± 2 days of age post-adult moult, when used in trials. Spermatophores of test males were removed 24 h prior to each trial to ensure that all males had fresh spermatophores and that spermatophore age would not be factor in female receptivity (Reinhardt & Siva-Jothy 2005). Female receptivity to a male was inferred if the female mounted the male, a prerequisite for spermatophore transfer. Trials took place in the male's container after removal of the water vial and shelter. Once the female was placed into the container, males were given 10 min to start courtship. If the male courted, the female was given 5 min in which to mount the male; mountings were interrupted prior to actual spermatophore transfer to eliminate the possibility of male accessory gland proteins influencing subsequent female behaviour (Loher & Edson 1973; Destephano et al. 1982; Murtaugh & Denlinger 1987; Loher et al. 1993; but see Fleischman & Sakaluk 2004; Green & Tregenza 2009). Females were tested twice each, with different males, and each male was used only once. The female's second trial took place 24 h after the first; 24 h was chosen because female *G. texensis* average 9.8 matings in 10 days in the laboratory (Solymar & Cade 1990). All trials were conducted at 19.5–23.7 °C. Females were tested in the second trial regardless of whether they mounted the first male. Neither *G. texensis* nor *G. rubens* males produced calling songs during trials.

To help separate the effects of female choice and mate sampling from the effects of male behaviour, during each trial we also recorded (1) latency to courtship by males, measured from the time of antennal contact between the pair until the male began his courtship song, (2) duration of courtship and (3) instances of male aggression towards females. Aggression was scored if males used aggressive song during interaction with the female, attacked the female by chasing her with flared mandibles, or bit the female. Latency to courtship indicates the degree of male interest in females; duration of courtship and male aggression towards females might both be affected by female response. Duration of courtship might be short either because the female mounts the male, or because the male loses interest in the female. Male aggression towards females might reflect a male's lack of sexual interest in the female, or a male's frustration due to lack of female receptivity to courtship. To help interpret the results for female behaviour, we present the results for male behaviour first.

RESULTS

Males

Both *G. rubens* and *G. texensis* males readily courted both conspecific and heterospecific females. Neither species preferentially courted conspecifics (generalized linear model with a binomial fit, hereafter binomial GLM: $\chi^2_2 = 1.12$, $N = 438$, $P = 0.57$).

For instances of aggression against females, the results were quite different. Male *G. rubens* were overall more aggressive than male *G. texensis* (binomial GLM: $\chi^2_2 = 47.43$, $P < 0.001$). Males of both species showed significantly higher rates of aggressive behaviour towards heterospecific females than towards conspecific females (binomial GLM blocked by male species *G. rubens* males: $\chi^2_1 = 5.78$, $P = 0.016$; *G. texensis* males: $\chi^2_1 = 9.62$, $P = 0.002$).

Results for male latency to courtship and duration of courtship are shown in Table 1; comparisons were made via nonparametric Kruskal–Wallis ANOVA. Courtship latencies of male *G. texensis* towards conspecific females were significantly shorter than those towards heterospecific females ($H_1 = 5.79$, $P < 0.016$) and significantly shorter than those of male *G. rubens* towards heterospecific females ($H_1 = 12.31$, $P < 0.001$). *Gryllus rubens* and *G. texensis* males did not differ in their latency to court female *G. rubens* ($H_1 = 0.02$, $P = 0.882$), and male *G. rubens* did not differ in their latency to court females of either species ($H_1 = 0.86$, $P = 0.354$). Courtship duration of conspecific and heterospecific females did not differ significantly between males of either species, although male *G. texensis* tended to spend more time courting heterospecific females than they did conspecific females ($H_1 = 3.67$, $P = 0.055$; Table 1). Overall, male *G. texensis* courted females of both species longer than did male *G. rubens* (*G. rubens* females: $H_1 = 10.29$, $P = 0.001$; *G. texensis* females: $H_1 = 8.30$, $P = 0.004$).

Table 1
Male courtship directed towards females of each species

	Male species	Female species		Comparison between females
		<i>G. rubens</i>	<i>G. texensis</i>	
Latency to courtship	<i>G. rubens</i>	96 (47–143)	99 (67–162)	$H_1 = 0.86$, $P = 0.354$ $H_1 = 5.79$, $P = 0.016$
	<i>G. texensis</i>	77 (51–174)	62 (36–97)	
Comparison between males		$H_1 = 0.02$, $P = 0.882$	$H_1 = 12.31$, $P < 0.001$	
Duration of courtship	<i>G. rubens</i>	13 (0–76)	3 (0–55)	$H_1 = 2.11$, $P = 0.147$ $H_1 = 3.67$, $P = 0.055$
	<i>G. texensis</i>	45 (0–348)	13 (0–36)	
Comparison between males		$H_1 = 10.29$, $P = 0.001$	$H_1 = 8.30$, $P = 0.004$	

Median latency to courtship and courtship duration (both in seconds) are shown. Interquartile ranges are given in parentheses. Statistical comparisons were made via nonparametric Kruskal–Wallis ANOVA.

Females

Table 2 summarizes female mating trial results. A three-way contingency table (female species (*rubens*/*texensis*), male species (conspecific/heterospecific), mount (yes/no)) demonstrated significant differences in female mating behaviour and preference for conspecific versus heterospecific first males ($G_4^2 = 35.1$, $P < 0.0001$). We then used 2×2 contingency tables with Bonferroni corrected $\alpha = 0.0071$ to examine differences within the larger table. For *G. rubens* females, there was no significant difference in frequency of mounting conspecific males versus heterospecific males ($G_1^2 = 2.62$, $P = 0.105$). However, female *G. texensis* mounted conspecific males in significantly more trials than they mounted heterospecific males ($G_1^2 = 29.89$, $P < 0.0001$). Therefore, female *G. rubens* appeared to show no preference for conspecifics when they had no prior experience, whereas female *G. texensis* did show a preference for conspecific males when they lacked prior mating experience.

Next, we examined female behaviour in the second of the paired trials. We used a three-way contingency table to determine the effect of prior experience with conspecific males on female mating decisions. Female behaviour was affected by prior experience, and this effect differed significantly between species ($G_4^2 = 19.54$, $P = 0.0006$). To examine these differences, we used 2×2 contingency tables (with Bonferroni corrected $\alpha = 0.0071$). For second trials in which the female's first experience was with a conspecific male, female *G. rubens* mounted a significantly higher proportion of heterospecific males than they did conspecific males ($G_1^2 = 7.16$, $P = 0.007$; Fig. 1). Female *G. texensis* showed the opposite trend: they mounted a significantly higher proportion of conspecific males than they did heterospecific males ($G_1^2 = 8.80$, $P = 0.003$; Fig. 1). When females had prior experience with conspecific males, *G. rubens* females preferred heterospecific males, whereas *G. texensis* preferred conspecific males.

For second trials in which the female's first experience was with a heterospecific male, neither species of female showed a preference for conspecific or heterospecific males ($G_3^2 = 6.91$, $P = 0.075$).

To examine whether the preference for *G. texensis* males by females of both species was partly due to the high levels of aggressive behaviour shown by *G. rubens*, we reran the analyses excluding all trials in which males showed aggressive behaviours towards females. Our results did not differ from those reported above.

DISCUSSION

Some of our results were as predicted, and others were unexpected. Overall, we found support for our predictions of how sequential mate choice affects female mating preferences. Female *G. texensis*, the previously more abundant species, followed our predictions closely.

Naïve females preferred conspecifics. Females that had prior mating experience with conspecifics were reluctant to trade down in quality with subsequent males, and showed strong preferences for conspecifics in the second trial. When *G. texensis* females had prior mating experience with heterospecific males, their choosiness was relaxed and they showed no preference for either conspecific or heterospecific males. This species of cricket behaved as predicted in all situations.

In contrast, the behaviour of the derived and previously less abundant *G. rubens* revealed a more convoluted picture. We expected female *G. rubens* to be less choosy than female *G. texensis*, given our current understanding of their long-term evolutionary past (Gray et al. 2008). However, we did not expect lability in *G. rubens* mate preference to result in a preference for heterospecific males. *Gryllus rubens* females fit our prediction of being more amenable to heterospecific crosses (e.g. naïve females showed no preference) and showed relaxed preference when their prior experience was with heterospecific males. However, females that had prior experience with conspecific males also showed a preference for heterospecific males. Given that this result contradicts previous work (Gray 2005), we are unsure how best to interpret this finding. *Gryllus texensis* male courtship song is delivered at a higher rate than is *G. rubens* courtship song (Fitzpatrick & Gray 2001). Because females of many, if not most, species prefer faster, more energetic sexual signals (Ryan & Keddy-Hector 1992), it is possible that female *G. rubens* genuinely do prefer faster *G. texensis*-like courtship song. If faster courtship song indicates male quality within a species, then this would be a nice example of the potential for within-species sexual selection to result in heterospecific preference (Pfennig 1998). Although we are not advocating this interpretation, we also cannot rule it out: *G. rubens* courtship song has not been studied with respect to a quality signalling function, and results with other *Gryllus* species are mixed. Courtship song of *G. texensis* does not appear to signal male nutritional history (Gray & Eckhardt 2001), whereas courtship song of *Gryllus bimaculatus* is related to the encapsulation ability of the immune system (Rantala & Kortet 2003).

Our present results could be interpreted to suggest that hybridization between these species in sympatry is likely, but results of morphological, bioacoustic and molecular genetic studies all suggest that natural hybridization is rare (Gray & Cade 2000; Gray et al. 2001, 2008; Izzo & Gray 2004). It is important to bear in mind that the courtship interactions described in this study normally occur in the wild following female phonotaxis to male calling song. The calling songs are species specific, and females of both species have a strong preference for conspecific calling song (Gray & Cade 2000). Thus, the opportunity for hybridization based solely on courtship interactions is probably limited. Although conspecific sperm precedence has not been investigated in *G. rubens* and *G. texensis*, conspecific sperm precedence may contribute to reproductive isolation in other cricket

Table 2
Comparison of mounting behaviour for each species of female during the first and second trials

Female species	1st trial		2nd trial		P
	Male species	Males mounted	Male species	Males mounted	
<i>G. rubens</i>	Conspecific	(30/58) 51.7%	Conspecific	(22/39) 56.4%	0.007
	Heterospecific	(31/46) 67.4%	Heterospecific	(17/19) 89.5%	
			Conspecific	(14/25) 56.0%	0.685
			Heterospecific	(13/21) 61.9%	
Con vs Het $P=0.105$					
<i>G. texensis</i>	Conspecific	(61/75) 81.3%	Conspecific	(39/59) 66.1%	0.003
	Heterospecific	(12/40) 30.0%	Heterospecific	(4/16) 25.0%	
			Conspecific	(8/19) 42.1%	0.11
			Heterospecific	(4/21) 19.0%	
Con vs Het $P<0.0001$					

2×2 contingency tables were used for statistical results. Alpha-criterion used was $\alpha \leq 0.0071$ (Bonferroni correction for seven tests).

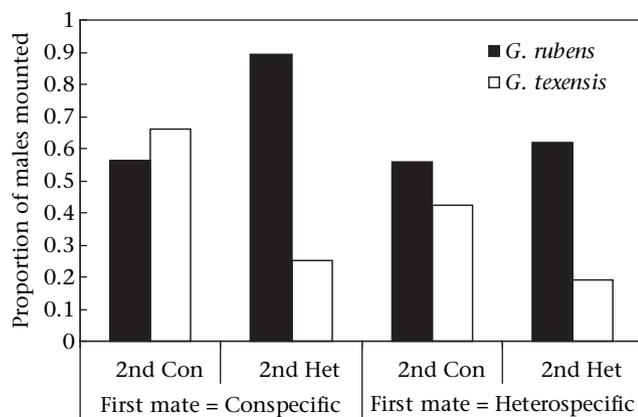


Figure 1. Proportion of second males mounted for each species of female, broken down by the species of the first male.

species (Howard et al. 1998; Britch et al. 2007). It is probably typical that reproductive isolation involves multiple signals and/or multiple stages during mating interactions (Hankison & Morris 2003; Mendelson et al. 2007; Matsubayashi & Katakura 2009).

In summary, our results illustrate several issues of general importance in mate choice studies, as well as several issues more specific to this pair of sister taxa. First, our findings indicate that results of sequential choice trials can differ significantly from results of simultaneous or no-choice trials. For example, in single trial no-choice tests, Gray (2005) found that female *G. rubens* preferred conspecific courtship to heterospecific courtship, whereas here we show that *G. rubens* females appear to prefer heterospecific courtship (in the absence of phonotaxis to male calling song). Second, mating interactions between species were not symmetrical (see also Wirtz 1999). Our results for *G. rubens* and *G. texensis* differed in many ways: *G. rubens* males were more aggressive towards females during courtship, *G. texensis* males were more likely to court any and all females than were *G. rubens* males, and *G. texensis* females showed strong conspecific mate preference, but female *G. rubens* did not. If asymmetry is true generally, then it may be difficult to generalize results for a single species to congeneric species, even closely related ones. Third, prior-male effects may interfere with female mate sampling, especially if some of the males encountered are heterospecifics. The potential conflict between species and mate quality recognition (Pfennig 1998) may be exacerbated by sequential sampling of potential mates. In most cases we have a priori expectation that conspecific mates are higher quality than heterospecifics, although results of some studies indicate that adaptive hybridization could be a possible confounding factor (Abt & Reyer 1993; Pfennig 2007). Theoretical modelling could clarify the utility of threshold versus prior-male decision rules when a significant percentage of encountered males are likely to be heterospecific. Fourth, mating interactions are a result of both male and female behaviour, and the mating behaviour of one sex may often be influenced by the mating behaviour of the other sex. Although this is fairly obvious, female mate choice researchers often tacitly assume that males are universally willing, and that female mate preferences are the sole (or at least primary) determinant of male mating success. Here we show significant levels of male disregard of females, especially by *G. rubens* males. As courtship interactions are iterative, male behavioural strategies may switch from active courtship to active aggression given female disinterest, and these male behaviours can greatly affect female preference within a system. This highlights the importance of examining both male and female behaviours to gain an overall view of how sexual selection operates within a taxon.

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