1 **Testing female preferences under more natural conditions: a case study on a fiddler crab**

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3 Regina Vega-Trejo^{1,*} and Patricia R. Y. Backwell¹

4 ¹Division of Ecology and Evolution, Research School of Biology, The Australian National

5 University, Acton, ACT 2601, Australia

6 *Corresponding author; Regina Vega-Trejo, e-mail: reginavegatrejo@gmail.com

7 Ph: +61 2 6125 3828

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Abstract

Mate choice is often affected by multiple factors and there are often trade-offs associated with choosing a mate. Additionally, experiments that test for mate preferences usually rely on simple two-choice tests. These tests are, however, often less complex than the scenarios that individuals face in natural populations. Here we test female choice in the fiddler crab *Uca mjoebergi*. We looked at female preference for wave rates and proximity to males in simple two-choice tests. We then mimicked a more natural choice scenario, where females faced a cluster of six courting males that differed in their distance from the female as well as in their wave rate. In addition, we tested whether female preferences under these more complex conditions were affected by the risk of predation. We found a preference for faster wave rates and closer males in two-choice tests. The preference for closer males was, however, only evident when the difference in distance was large (15cm), not when it was small (3cm). When females chose between six males they preferred the males that waved faster, even if they were further away. We did not, however, find any difference in female choice when a simulated predator was present or absent. By examining a more realistic set of options that females face we can paint a better picture of how females trade-off costs and benefits during mate choice.

Keywords: two-choice, predation, sexual selection

Significant statement

Mate choice experiments often rely on two-choice tests. Mate choice, however, is often more complex under totally natural conditions. Using a two-choice experiment we show that female fiddler crabs *Uca mjoebergi* show a preference for faster wave rates and closer

males. Under a more natural choice scenario, when choosing between six males, females preferred to travel longer to reach faster waving males. We found that female responses did not differ when a predator was present or absent. Designing choice experiments to more accurately mimic natural conditions will allow assessing trade-offs that occur in mate choice.

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Introduction

Mating preferences and preferred mate traits are often constrained by costs associated with mate choice. That is, females are often choosy when looking for potential mates and show a preference for particular sexual ornaments and signals (Jennions and Petrie 1997; Bonachea and Ryan 2011). However, there are costs associated with male sampling that can influence female mating preferences (Bakker and Milinski 1991; Cotton et al. 2006). This is especially true in species where females visit multiple males before selecting a mate. The costs of moving between potential mates are often high and the benefits of finding a high-quality male must be weighed with the costs incurred by an increased sampling effort (Real 1990; Bonachea and Ryan 2011; Lindström and Lehtonen 2013). This trade-off between mate suitability and costs of sampling affects both sampling patterns and final mate choice (Backwell and Passmore 1996).

In the 1980s and 1990s mate choice studies aimed to establish whether variation in mating success was non random, determine whether individuals vary in their mate choice, determine the range of trait values being chosen and figure out how individuals find the best potential mates (Real 1990; Andersson 1994; Jennions and Petrie 1997). To achieve these aims, relatively simple experimental designs involving mostly a two choice design were employed. For example, two-choice phonotaxis experiments were used to show that female frogs had preferences for certain call variants, which were dependant on the surrounding environment and neighbours that a female was exposed to (Dyson and Passmore 1988; Backwell and Passmore 1990). Over the past 25 years, we have amassed a vast set of studies showing that mate choice is a complex process. Mate choice is plastic (Wacker et al. 2016), and it can involve multiple traits (Chenoweth and Blows 2006; Head et al. 2016), it often

changes over time (Kahn et al. 2013), it can depend on voyeurism (Auld and Godin 2015), and it can even be irrational (Lea and Ryan 2015).

Although we understand many of the complexities of mate choice, we are lagging in our experimental designs for examining choices. Even in the early days of phonotaxis experiments in frogs we knew that the preferences females exhibited in two-choice trials did not always translate into the natural setting (Telford et al. 1989) because the conditions of choice were more complex than those in a highly controlled two-choice test. This has recently become an issue that is again under discussion (Dougherty and Shuker 2015; Rowe and Arnqvist 2015): *“we should take great care in designing studies of mate choice if our goal is to project our conclusions to natural populations”* (Rowe and Arnqvist 2015).

Controlled experimental studies of mate choice are valuable as a supplement to correlational information from natural observations of male (or female) mating success, but we suggest that it is necessary to use experimental designs that more closely mimic the context in which mate choice naturally occurs (see Rowe and Arnqvist 2015). Studies that test female preferences under more realistic settings than two-choice trials could allow us to detect more subtle effects and interactions between factors. For instance, the costs of moving between potential mates are often high and the benefits of finding a high-quality male must be weighed with the costs incurred by an increased sampling effort (Real 1990; Bonachea and Ryan 2011; Lindström and Lehtonen 2013). For example, female choosiness for quality song can decrease in female crickets when there is perceived predation risk (Atwell and Wagner 2015). Thus, more complex experimental settings could ultimately allow us to better translate experimental results into the natural setting and allow us to get a better grasp of trade-offs.

An ideal species to examine female mate choice under more natural conditions is the fiddler crab, *Uca mjoebergi*. We already know much about the natural behaviour of this species, and we have good information on their mate preferences in simple two-choice tests using robotic crabs (e.g. Booksmythe et al. 2008; Reaney 2009; Holman et al. 2014). Here, we examine female mating preferences under more complex conditions involving choice between six males at different distances from the female and either in the presence or absence of a predator.

Study System

Uca mjoebergi is a small fiddler crab (<2 cm carapace width) that lives in high density (approximate density 37 ± 17 crabs/m²; R. Slatyer, L. T. Reaney & P. R. Y. Backwell, unpublished data), mixed-sex populations on inter-tidal mudflats (Reaney and Backwell 2007). Each individual defends a territory with a central burrow that is a heat sink, a water source, a mating and incubation site, and a place to escape from predators (Reaney and Backwell 2007). The burrow is surrounded by a small area (± 10 cm diameter) that individuals use for feeding and courting. Mating occurs over 5–9 days in each 14–17 day tidal cycle (Reaney and Backwell 2007). Once a female is ready to mate she leaves her territory and wanders through the population of waving males, visiting an average of three males (briefly entering their burrows) before selecting a mate. Mating occurs in the male's burrow where he guards her for a few days until she releases her eggs onto her pleopods (Reaney and Backwell 2007). The male then leaves and seals the female into the burrow, where she remains for approximately 20 days to incubate her eggs. Females re-emerge at a nocturnal spring tide to release their pelagic larvae into the water (Reaney and Backwell 2007).

The natural mating behaviour of this species is complex. Female mating preferences vary both temporally and spatially. Females living in the high inter-tidal zone change their mating preference for male size over the 9-day mating period every semi-lunar cycle: early mating females select larger males with cooler burrows, slowing embryonic development; females mating later, select smaller males with warmer burrows, accelerating development (Reaney and Backwell 2007; Milner et al. 2010). Females living lower in the inter-tidal zone, however, do not show this temporal variation: they select the same sized males throughout the mating period. It is only in the high inter-tidal zone, at the start of the fortnightly mating period, that large size confers a male mating advantage (Clark and Backwell 2015).

Females select mates from small clusters of courting males that surround her as she moves through the population, and they choose males with higher wave rates than those of nearby male competitors (Callander et al. 2012). Observations of natural mate-searching females also suggest that they are less selective (bypass fewer males) when the risk of predation is higher (Booksmythe et al. 2008).

From two-choice robotic crab trials we know that females prefer males with larger claws (Reaney 2009; Milner et al. 2010) and males who wave at faster rates (Reaney et al. 2008; Reaney 2009). Females also prefer males that produce leading signals (Reaney et al. 2008), prefer closer males (Booksmythe et al. 2008), have UV cues on their claws (Detto and Backwell 2009), and have the conspecific yellow claw colouration (Detto et al. 2006). Moreover, females avoid males that display from elevated positions (Holman et al. 2014) and female preferences are strongest when competing males are close to each other (Peso et al. 2014).

In this study, we aim to confirm that females prefer (i) faster wave rates and (ii) nearer males under simple two-choice experimental conditions. We then combine these two factors to create a more natural choice scenario where females are faced with a cluster of six courting males that differ in distance from the female as well as wave rate. We also test whether female preferences under these more complex conditions are affected by the risk of predation. Given that females prefer males that are closer and males with faster wave rates in two-choice trials, we predicted that females would have to trade-off males that were far with fast wave rates with males that were close with slow wave rates under predation risk.

Methods

We examined female mating preferences in a population of *Uca mjoebergi* at East Point Reserve, Darwin, Australia (12.41°N, 130.83°E) in November 2015. The experimental test arena was set up in a clearing in the mangrove trees, on the edge of the study population. We tested female preferences using robotic crabs consisting of a twin-cam motor that moves a small metal arm in a waving motion, exactly mimicking the mate attraction wave. The motor is remotely controlled to regulate the exact timing of each wave using custom designed software (see Reaney et al. 2008; Booksmythe et al. 2011; Holman et al. 2014 for further details of the robotic crabs). The motor was placed under the testing arena and the metal arm protruded through the arena floor. The arm had a plaster replica of *U. mjoebergi* claw attached to it. For all trials, we used replicas of the same claw, each measuring 24 mm. The surface of the test arena was covered by a 1 cm thick layer of mudflat sediment. We conducted two-choice tests on an arena that measured 40 x 60cm and six-choice tests on an arena that measured 60 x 60 cm. For all experiments, the order and side of stimuli

presentation was randomised. It was not possible to record data blind because we focused on the female's response.

We captured mate-searching females as they wandered through the population of courting males. These females are easily identified as they approach and enter the burrows of waving males. We placed the captured females in the shade in a cup with a small amount of seawater. The females were released after they were tested so that they could continue mate-searching. Females naturally visit numerous males before selecting a mate, so it is not unreasonable to test them in multiple trials. Before each trial, the female was measured (carapace width) and placed in a small, inverted, transparent cup on the arena surface. The robotic crabs were allowed to produce three full cycles of waving before the female was remotely released. A choice was scored when the female directly approached a claw and moved to within 2cm of it. Trials were discarded if the female darted, ran to the edge of the area, or remained stationary for >3 mins. Each female was retested up to a maximum of three times and excluded if they were all non-responses. Females were tested in multiple trials, but were only tested in the same type of trial once (i.e. two-choice vs six-choice). For the predation/no predation experiments, females were only tested in one of the trials to prevent past experience of predation affecting the trial outcome.

Two-choice trials: i) Fast vs slow wave rates, but same distance: we determined female preferences for fast over slow wave rates by giving the female a choice between two robots placed 5cm apart and 20 cm directly in front of the female release point. One robot waved at 4.2 waves/min (slow rate) and the other waved at 16.8 waves/min (fast rate). ii) Close vs far (small and large differences), but same wave rate: we determined preferences for closer over further males by giving the females a choice of two identical robots, both waving at the fast rate (16.8 waves/min) and both directly facing the female, but differing in their

distances from the female release point. In one set of trials, one robot was 3 cm further from the female (14 cm vs. 17 cm); and in another set of trials, one robot was 15cm further away (14 cm vs. 29 cm).

Six-choice trials: we presented females with a choice of six robotic crabs, each one 3 cm further away than the next (14 cm; 17 cm; 20 cm; 23 cm; 26 cm; 29 cm). The two nearest robots both waved at the slow rate (4.2 waves/min). The middle two robots both waved at the medium wave rate (8.4 waves/min). The furthest two robots waved at the fast rate (16.8 waves/min). Fig. 1 gives a graphical representation of the experimental arena.

We repeated the six-choice trials: once with a simulated predator and once without. The simulated predator consisted of a life-size model of a bird painted black and attached to a zip line that ran above the test arena (see Booksmythe et al. 2008 for a similar design). The bird was released at the same time as the female was released, and it flew at a decreasing height (20cm – 10cm) directly above the central line between the female release point and the robotic crabs.

Statistical analyses: To determine female preference for i) fast vs slow wave rates, but same distance (N = 20 trials) and ii) close vs far (small and large differences), but same wave rate (N = 20 trials for small differences, N = 20 trials for large differences) we performed binomial tests.

For our six-choice trials we first compared whether female preference differed from a random distribution using a chi-squared test. We did this separately for each set of trials: without a predator (N = 40 trials) and with a predator (N = 40 trials). Since our two-choice trials revealed that small differences in distance between males did not influence female

choice (see results below), we combined responses to pairs of robots with the same wave rates. We then tested whether they preferred certain wave rates using binomial tests. To control for multiple testing, we used false discovery rate correction (FDR; Benjamini and Hochberg 1995).

Using the combined responses of pairs of robots with the same wave rates, we tested whether female preference was different with or without a predator using a chi squared test. To compare trials with and without a simulated predator for specific wave rate combinations we performed a multinomial logistic regression using the package *nnet* using R version 3.2.4 (R Development Core Team 2012). We ran the multinomial regression twice, each time setting the reference category to a different choice option (i.e. near/slow and far/fast) to obtain p-values for each of the possible preference combinations. This test looks at whether the risk of predation influences how females choose between the different wave rates. We additionally compared the number of times a female approached each of the wave rates across trials that differed in predation risk using binomial tests.

Results

Two-choice trials: i) females showed a very strong preference for robotic crabs that waved at a faster rate (16.8 versus 4.2 waves/min) when they were both 20 cm away from the female release point. All 20 females approached the faster waving robot (binomial test: $P < 0.001$).

ii) When both robots waved at the same rate (16.8 waves/min), but were different distances from the release point, the females showed no preference for either signal when the difference in distance was small (14 cm and 17 cm away from the female, 13:7 responses,

binomial $P = 0.26$). When the difference in distance was large, however, females preferentially approached the closer robot (14 cm vs 29 cm, 15:5, binomial $P = 0.04$).

Six-choice trials without a simulated predator: when presented with an array of six robotic crabs, each 3 cm further away from the female release point and differing in wave rates, the number of responses was higher for far away males with a fast wave rate (Fig. 2a).

The number of responses to each of the six robots differed from a random distribution ($\chi^2 = 21.74$, d.f. = 5, $P < 0.001$). Combining the responses to pairs of robots with the same wave rates, we found a difference in female responses ($\chi^2 = 18.49$, d.f. = 2, $P < 0.001$) to the slow, medium and fast wave rates. They were more likely to approach the fast waving robots (over the medium or slow waving robots) even though they were further away (Fig 2a). There was no difference in the number of approaches to the medium and slow waving robots. Binomial tests: slow vs fast $P_{FDR} < 0.006$; medium vs fast $P_{FDR} = 0.03$; slow vs medium $P_{FDR} = 0.22$).

Six-choice trials with a simulated predator: the number of responses was higher for far away males with a fast wave rate (Fig. 2b). However, unlike the non-predator trials, the number of responses to each of the six robots did not differ from a random distribution ($\chi^2 = 7.98$, d.f. = 5, $P = 0.16$). Binomial tests: slow vs fast $P_{FDR} = 0.65$; medium vs fast $P_{FDR} = 0.04$; slow vs medium $P_{FDR} = 0.17$).

Comparison of trials with and without a simulated predator: The presence of a simulated predator had no overall effect on the distribution of female responses across slow, medium, and fast male wave rates ($\chi^2 = 6.15$, $P = 0.05$). There was also no effect of predation risk on the difference in female responses to specific combinations of wave rates (multinomial logistic regression: slow vs medium $P = 0.21$, medium vs fast $P = 0.99$, slow vs fast $P = 0.144$).

Finally, we found no difference in the number of approaches to each of the wave rates when a predator was present or absent (Binomial tests: slow-with predator vs slow-without predator $P = 0.27$; medium- with predator vs medium- without predator $P = 0.56$; fast- with predator vs fast- without predator $P = 0.56$).

Discussion

The results from the two-choice trials are clear: (i) females strongly prefer males with a faster wave rate; (ii) females prefer closer males when the difference in distance is 15 cm; but (iii) they showed no preference for proximity when the more distant stimulus was only 3 cm further away. In the more complex six-choice trials, the females had to balance two variables: wave rate and distance. Under this scenario females preferred far away males with fast wave rates over close males with slow wave rates. Based on the results from our two-choice trials, we suggest that females prefer males with a higher wave rate, in spite of having to travel a further distance.

Although our experimental design did not allow us to tease apart the effects of distance and wave rate, when the females were exposed to a risk of predation, females appeared to be less willing to travel long distances to reach a male with a high wave rate, although this effect is likely to be weak. When a simulated predator was present, female choice for males was no longer significantly different from random, but neither was it significantly different to female choice when there was no predator present. This is similar to a study in pipefish which found that the presence of a predator made mating random (Berglund 1993). In our study, females still approached the distant males with the fast wave rates more often than the closer males with the slower wave rates when a predator was present, but this choice

was only marginally significant and so we treat our observation with caution given its weak statistical support.

Females often need to weigh the benefits of choosing attractive mates with costs of predation that can be associated with mate searching and assessment (e.g. Booksmythe et al. 2008; Atwell and Wagner 2015) or even with mating with certain males (e.g. Forsgren 1992; Johnson and Basolo 2003). When there is a risk of predation females are expected to reduce their overall searching time by evaluating fewer males, assessing each male for less time, and discriminating less between males (Pomiankowski 1987; Real 1990; Hedrick and Dill 1993). Additionally, females can alter their mate preference (e.g. Gong and Gibson 1996; Evans et al. 2004; Kim et al. 2009; Zhu et al. 2012). Decreased investment in mate choice in the presence of a simulated predator may explain why female mate choice during our predation trials did not differ from a random distribution. Despite a shift in mate choice towards random mating, mate choice did not differ between the predation and no predation trials. This result might arise because the degree to which predation can weaken female preferences is constrained between maximizing their choice (i.e. choice when no predators are present) and choosing at random, thus effects might be expected to be small and large sample sizes may be needed to detect the weakened preference.

Under natural conditions, a mate-searching female *Uca mjoebergi* is more likely to approach a male whose wave rate is faster than his close neighbours (this study and Callander et al. 2012). Assessing the added effect of distance is difficult under natural conditions since females do not always approach males in a straight line, not all males are directly facing her, and males change their wave rates and relative proximity to the female as she moves towards them. We suggest that there is still a use for experimental choice trials, but that

they would be even more useful if they better reflected some of the complexity found in the field.

The most common design for testing female preferences is the simple two-choice test in an experimental arena that controls for all but one variable (Wagner 1998; see Velásquez et al. 2015; Yasumiba et al. 2015; Zhu et al. 2016 for recent examples), but there are increasingly examples of more complex choice tests (e.g. Lea and Ryan 2015; Schwartz et al. 2016). There has been a recent call for a plurality of approaches including highly controlled two-choice trials, more natural test designs, and experiments that assess mate choice in more natural social and environmental contexts (Dougherty and Shuker 2015; Rowe and Arnqvist 2015). For example, whether a female uses one or multiple cues to select a male might depend on the costs associated with the assessment of males (Fawcett and Johnstone 2003). More complicated experimental designs can, not only demonstrate which cues are important in mate choice, but can help estimate the relative importance of indirect and direct benefits of mate choice (Chenoweth and Blows 2006; Owens 2006). By gaining insight into the effects of social and environmental variables on the strength of mate choice, we will better understand how signals evolve (Rowe and Arnqvist 2015).

Ethical approval

This work was performed under an ethics approval permit (A2015/54) from the Australian National University Animal Experimentation Ethics Committee (ANUAEEC)

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Fig. 1 Graphical representation of the experimental set up. Males were placed at 14, 17, 20, 23, 26, and 29 cm from the female release point. Wave rate increased with distance. S = slow wave rate (4.2 waves/min), M = medium wave rate (8.4 waves/min), F = fast wave rate (16.8 waves/min)

Fig. 2 Number of observations of females choosing either slow (S), medium (M), or fast (F) wave rates when there was no simulated predator (a) and when there was a simulated predator (b). Within each bar, the lighter portion is the number of responses to the nearer of the two stimuli and the darker portion is the number of responses to the further of the two stimuli.