Strong, but incomplete, mate choice discrimination between two closely related species of paper wasp

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Paper wasps (genus *Polistes*) are one of the most species-rich genera of social insect. Prior studies have found that male coloration, male colour pattern, territory choice and female caste are potential drivers of intraspecific mate choice in paper wasps. However, there has been no formal assessment of interspecific mate choice in this group; therefore, the mechanism driving diversification in paper wasps remains an open question. In this study, we measured interspecific and intraspecific mating behaviour between two closely related species of paper wasps, *Polistes fuscatus* and *Polistes metricus*. These two species have ample opportunity to interbreed because *P. fuscatus* and *P. metricus* forage, nest and mate in the same habitats. We tested the strength of reproductive isolation between these species using no-choice and choice mating trials. Our results show strong, symmetric, prezygotic isolation between *P. fuscatus* and *P. metricus*. Males discriminated between conspecifics and heterospecifics but attempted to mate with females of the other species in ~10% of heterospecific mating trials. Female wasps were more discriminating than males and probably evaluated species identity and male quality through visual or olfactory cues. We additionally report sexual dimorphism in *P. metricus* body size.

ADDITIONAL KEYWORDS: mate choice – *Polistes* – social insects – speciation.

INTRODUCTION

Social hymenopterans are a highly successful group of organisms. Surprisingly, the mechanisms that cause diversification in this group have received little attention (Boomsma *et al.*, 2005; Baer, 2014). One approach to studying the process of speciation is to identify reproductive boundaries between species and to test the strength of these boundaries (Coyne & Orr, 2004). Understanding reproductive boundaries in social hymenoptera is of interest because features of social insect mating systems predict that social insects should have high specificity in mating signals, which can promote speciation and diversification (Coyne & Orr, 2004). For example, populations of red-tailed bumblebees (*Bombus lapidarius*) show recent divergence in male marking secretions, an important cue for mate attraction (Lecocq *et al.*, 2013). However, male social insects lack energetically costly mating displays and rarely display male-specific

ornamentation characteristic of other systems with strong sexual selection (Boomsma *et al.*, 2005; Baer, 2014; Beani *et al.*, 2014), leaving the cause of diversification in this group an open question.

Both male and female social hymenoptera have highly skewed distribution in mating success. The majority of female social hymenoptera are non-reproductive workers. Only a small proportion of female social hymenoptera mate, and for primitively social species, such as paper wasps, many mated females join cooperative foundress associations and never reproduce (Reeve, 1991). Male *Polistes dominula* paper wasps can distinguish between non-reproductive workers and reproductive females (Cappa *et al.*, 2013). Mating in social hymenoptera typically occurs in leks or groups, characterized by strong male competition and female choice (Boomsma *et al.*, 2005; Beani *et al.*, 2014). Females are monogamous for most species of social hymenoptera (Strassmann, 2001). Male social hymenoptera do not provide parental care or nuptial gifts, with rare exceptions (see Beani *et al.*, 2014), but contribute only gametes to the offspring. As a result, a female is likely to choose to mate with a male solely

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based upon her assessment of male quality. Together, these features predict that social hymenopteran mating signals should show high specificity.

Polistes paper wasps are one of the most species-rich genera of social insects, making this an interesting candidate group in which to investigate social insect diversification. There are > 200 recognized species of paper wasp (Carpenter, 1996). Species display wide variation in body and facial coloration both within and between species (Carpenter, 1996; Tibbetts, 2004; de Souza *et al.*, 2017b). Paper wasp species often have overlapping ranges, but there is little ecological differentiation among paper wasps (Richter, 2000; Buck *et al.*, 2008). Together, these observations suggest that diversification in these species might be driven or reinforced by specificity in mating signals. In support of this prediction, coloration has been shown to be an important signal for mate choice within species. Male stenogastrine wasps have sexually dimorphic stripes on their tergites, which are displayed during territorial competition and mating (Beani & Turillazzi, 1999). In *P. dominula*, the size of a yellow abdominal marking influences female mating choice and male–male competition (Izzo & Tibbetts, 2012; de Souza *et al.*, 2017a). Likewise, black facial pigmentation and yellow abdominal spots can impact the choice of sexual partner for *Polistes simillimus* (de Souza *et al.*, 2014), and these markings may be honest signals of male quality (de Souza *et al.*, 2018).

In temperate latitudes, paper wasp colonies are started by a single wasp or by a small group of female wasps (foundress association) in the spring (Sheehan *et al.*, 2015). Colonies produce female worker offspring until mid-summer, when nests transition to rearing males and reproductive females (gynes). Males leave their natal nests to gather at leks or defend small contiguous territories near the tops of vertical structures, such as trees, buildings or telephone poles (Matthes-Sears & Alcock, 1986; Beani & Turillazzi, 1988, 1990a, b; Beani *et al.*, 1992; Polak, 1993; Beani & Zaccaroni, 2015). Mating occurs when females approach lekking sites. However, smaller males may attempt to mate opportunistically with females near foraging areas or flight paths (Post & Jeanne, 1983; Beani & Turillazzi, 1988; Polak, 1993). Mating territories commonly contain multiple species of paper wasp (Beani & Turillazzi, 1990b; Reed & Landolt, 1991). As a result of this overlap, unsuccessful heterospecific mating attempts have been observed in the wild (Post & Jeanne, 1983; Reed & Landolt 1991). These heterospecific mating attempts may be stimulated in part by a long-distance volatile pheromone produced by gynes, which has been shown to attract both conspecific and heterospecific males (Post & Jeanne, 1983; Ono & Sasaki, 1987; Reed & Landolt, 1990). After

mating, gynes diapause over winter, and the following spring they build new nests and rear the first group of worker offspring. Owing to the disproportionate female investment in offspring, paper wasp gynes might be choosier and more discriminating in mate choice than males.

This study assessed inter- and intraspecific mating behaviour between two species of paper wasp, *Polistes fuscatus* and *Polistes metricus*. These species are sympatric throughout most of their range, where they forage, nest and mate in similar habitats, offering ample opportunity for these species to interbreed (Miller *et al.*, 2018a). Quantifying reproductive isolation between *P. fuscatus* and *P. metricus* might be particularly interesting because these species exhibit only weak genetic divergence (Miller *et al.*, 2018b), suggesting that reproductive boundaries between these species might have formed recently or might still be in the process of forming.

MATERIAL AND METHODS

WASP COLLECTION

In September 2017, *P. fuscatus* nests ($N = 24$) were collected in New York (Schuyler and Thompson Counties) and *P. metricus* ($N = 11$) nests were collected in Maryland (Frederick and Montgomery Counties). *Polistes fuscatus* and *P. metricus* are sympatric in Maryland, but only *P. fuscatus* is found in New York. Adult wasps were housed in plastic containers, segregated by nest and sex. Pupae hatching in mid-September were either male or assumed to be reproductive females (gynes). Nests were monitored daily for eclosed wasps. Newly emerged wasps were immediately removed and housed with other nest members of the same sex to ensure that individuals had no prior exposure to members of the opposite sex. Wasps were kept in a 12 h–12 h light–dark cycle and fed water and sugar *ad libitum*.

MATING EXPERIMENT 1

Individuals were randomly assigned to mating trials ($N = 152$) in one of four groups: *P. fuscatus* female $\times$ *P. fuscatus* male; *P. metricus* female $\times$ *P. metricus* male; *P. fuscatus* female $\times$ *P. metricus* male; and *P. metricus* female $\times$ *P. fuscatus* male. Male and female wasps from the same nest were never paired. Wasps were individually housed during the course of the experiment to prevent the transmission of social or chemical information.

More females eclosed in the laboratory (*P. fuscatus* $N = 77$; *P. metricus* $N = 72$) than males (*P. fuscatus* $N = 37$; *P. metricus* $N = 23$). Owing to the discrepancy between the sexes, virgin females with no prior mating

experience were used in all trials, but laboratory-eclosed males were used multiple times, always with the same species of female. Using males in multiple mate choice trials is consistent with the experience of wasps in the wild, because male wasps will mate multiple times if given the opportunity, whereas female wasps typically mate only once (Hughes *et al.*, 2008).

Mating trials followed a no-choice design. A single virgin female and a single laboratory-eclosed male were introduced into a Petri dish containing a vial of water. Wasps were allowed to interact for 2 h and were weighed at the end of the trial. Trials were recorded with Sony HDR-CX405 video cameras. Males were allowed to recuperate for ≥ 24 h between trials. All trials were conducted between 10.00 and 16.00 h.

MEASUREMENT OF BEHAVIOUR

Behavioural interactions between males and females were quantified for the first 30 min of each trial. Male interest was determined by measuring the latency and frequency of three male behaviours required for mating (based on Beani & Turillazzi, 1988).

1. Approach/inspection: the male oriented his head and body in the direction of the female, walked towards the female and attempted to contact the female with his legs or antenna.
2. Mating attempt: the male mounted the female in preparation for mating.
3. Mating: genital linkage occurred for ≥ 10 s. Matings that occurred in the remaining 90 min of each trial were also recorded.

To assess female aggression, we counted the number of snaps (opening and closing of mandibles in the direction of the other wasp), bites and stings. Aggressive behaviours had a highly skewed distribution, driven by several extremely aggressive females. Therefore, we report aggression as the logarithm of the sum of the number of aggressive behaviours observed in each trial. All behaviours were scored using BORIS (v.6.2.2) by two observers blind to species identity.

MATING EXPERIMENT 2

There was a low proportion of successful matings in experiment 1. We therefore performed a second set of trials to test whether female receptivity increased when females were given a choice of mating partners. Mating trials used the same pairings as experiment 1 ($N = 99$). Experiment 2 began 2 days after the final experiment 1 trial. Most mating in experiment 1 occurred within the first 15 min of the trial; therefore, we reduced the length of mating trials to 30 min. Trials followed the same procedure as experiment 1, with the exception that each trial used two males.

Females in experiment 2 had been used previously in a single trial in experiment 1 but had not mated during that trial. Several males died before the start of experiment 2, either as a result of female aggression or owing to senescence. To increase the number of males in the experiment, additional wild-caught males collected on nests (*P. fuscatus* $N = 44$; *P. metricus* $N = 22$) or caught on the wing (*P. fuscatus* $N = 2$) were included. Wasps of both sexes were assigned to the same pairing as in experiment 1. For each trial, we report the latency for the first male to attempt to mate and the latency of the first copulation.

STATISTICAL ANALYSIS

Differences in male and female body size were assessed separately for each species using a linear mixed effects model with the nlme package (Pinheiro *et al.*, 2018), including nest of origin as a random effect. We used χ^2 tests used to measure the difference in the frequency of male behaviours between heterospecific and conspecific mating trials. Owing to the small number of counts in some groups, P -values were estimated using Monte Carlo simulations ($N = 2000$). Quantitative measurements of behaviour were not normally distributed; as a result, the non-parametric Kruskal–Wallis test was used to assess differences in these behaviours between conspecific and heterospecific trials. Following Coyne & Orr (1989), the strength of prezygotic reproductive isolation was estimated as: $1 - (\text{frequency of heterospecific mating} / \text{frequency of conspecific mating})$. All statistics analyses were calculated with R (v.3.4.3; R Core Team, 2018).

RESULTS

Polistes fuscatus and *P. metricus* were differentially sexual dimorphic in body weight. *Polistes metricus* females were smaller than *P. metricus* males (Fig. 1A; $F_{1,100} = 34.6$, $P < 0.0001$). *Polistes fuscatus* females were larger than *P. fuscatus* males, but this result was not statistically significant after correcting for nest ($F_{1,124} = 2.3$, $P = 0.13$). This resulted in females that were smaller than males in the *P. metricus* male $\times$ *P. fuscatus* female pairings, in comparison to other pairings (Fig. 1B).

EXPERIMENT 1

Male mating behaviour in no-choice trials was simple and identical for *P. fuscatus* and *P. metricus*. The male approached the female early in the trial. If she was receptive, the male mounted the female and mating occurred. Males did not perform any visible courtship

Figure 1. (A) body weight for *Polistes metricus* and *Polistes fuscatus* females and males. (B) the difference in body weight in experiment 1 between paired males and females from the four possible species pairings. Owing to the higher body weight of *P. metricus* (Met) males, females were smaller than males in the *P. metricus* male $\times$ *P. fuscatus* (Fus) female trials.

behaviour, indicating that female choice probably relied solely upon visual or chemical cues of male quality.

Males inspected females in 145 trials (96%). Males were equally likely to inspect conspecific and heterospecific females ($\chi^2 = 2.0$, $P = 0.75$) but were slower to inspect and spent less time inspecting females of the other species (Table 1; Fig. 2A, D; latency, $H_2 = 7.2$, $P = 0.007$; time, $H_2 = 33.9$, $P = 5.7 \times 10^{-9}$). Males mounted females (hereafter, mating 'attempt') in 40 of 152 trials (27%). Mating attempts were more likely to occur in conspecific trials ($\chi^2 = 41.7$, $P = 5 \times 10^{-4}$). Males attempted to mate with conspecifics more rapidly (Fig. 2B; latency, $H_2 = 7.2$, $P = 0.007$) and spent more time attempting to mate with conspecifics than heterospecifics (Fig. 2E; time mounting, $H_2 = 33.9$, $P = 5.7 \times 10^{-9}$). Mating occurred in 19 trials (13%). All matings were between conspecifics ($\chi^2 = 25.3$, $P = 5 \times 10^{-4}$). When mating occurred, the pair typically mated rapidly (median time, 308 s).

This experiment had a low rate of mating success, largely driven by female rejection of males. Females rejected males by performing aggressive behaviours towards the male, preventing males from mating, or by moving their abdomen to prevent genital linkage after mounting occurred. Females were more aggressive towards conspecific males ($H_2 = 5.5$, $P = 0.02$), but this difference was driven by the higher number of mating attempts by conspecific males than by heterospecific males. The highest rates of aggression were observed during trials between *P. fuscatus* females and *P. metricus* males (Fig. 2F).

There was uneven mating success among males. Only six of 19 males mated (32%) in *P. fuscatus* $\times$ *P. fuscatus* mating trials, with two males mating twice. Likewise, eight of 20 males mated (40%)

in *P. metricus* $\times$ *P. metricus* trials, with three males each mating twice. Mated *P. fuscatus* males were smaller than males that did not mate (paired t -test: $t = 2.5$, d.f. = 11.6, $P = 0.03$). Conversely, *P. metricus* males were larger in trials with successful mating, but this difference was not significant (paired t -test: $t = -0.86$, d.f. = 24.1, $P = 0.40$). The relative difference in male and female body size was not a predictor of mating success for *P. fuscatus* (paired t -test: $t = -1.1$, d.f. = 9.1, $P = 0.31$) or *P. metricus* (paired t -test: $t = -0.24$, d.f. = 32.4, $P = 0.81$).

EXPERIMENT 2

Males attempted to mount females in 34 trials (34%). As above, mating attempts were more likely to occur in conspecific trials (Table 2; $\chi^2 = 33.3$, $P = 5 \times 10^{-4}$), but there was no difference in the latency to attempt to mate between conspecific and heterospecific trials (Fig. 2G; $H_2 = 0.33$, $P = 0.56$). Mating occurred in 24 trials (25%). Mating was more likely to occur between conspecifics ($\chi^2 = 20.8$, $P = 5 \times 10^{-4}$); however, we observed two interspecific matings. One mating occurred between a *P. fuscatus* female and a *P. metricus* male, and one mating between a *P. metricus* female and a *P. fuscatus* male. In both trials, the female had a lower body weight than the male. The latency to mate was similar for conspecific and heterospecific trials (Fig. 2H; $H_2 = 0.05$, $P = 0.82$). The difference in the latency in mating attempts and mating observed between experiment 1 and experiment 2 was largely driven by a single heterospecific trial during which mating occurred almost immediately. The two female wasps that mated with heterospecifics were overwintered in the laboratory, but neither female produced offspring. One female died in the early spring before founding a

Table 1. Summary of results from mate choice experiment 1

Type	Trials	Approaches	Latency to approach	Attempts	Latency to attempt	Matings	Latency to mate	Female aggression
<i>P. fuscatus</i> female × <i>P. fuscatus</i> male	36	35	187.6 ± 60.6	16	234 ± 105.5	8	303 ± 181.4	2.1 ± 0.31
<i>P. fuscatus</i> female × <i>P. metricus</i> male	41	40	297 ± 64.8	4	703.9 ± 248.0	0	n.a.	1.43 ± 0.29
<i>P. metricus</i> female × <i>P. fuscatus</i> male	39	35	262.7 ± 56.9	0	n.a.	0	n.a.	1.11 ± 0.19
<i>P. metricus</i> female × <i>P. metricus</i> male	35	35	188.6 ± 43.9	20	313.4 ± 71.2	11	1626.4 ± 807.3	1.62 ± 0.20

n.a. indicates that no wasps performed the behavior. Approaches, attempts and matings refer to the number of trials during which a male performed that behaviour. Latency to approach, latency to attempt and latency to mate are the mean and standard error of the time it took a male to perform that behaviour during the trial (in seconds). Female aggression is the mean and standard error of the female aggression index (see Materials and methods).

nest. The second female joined a cooperative foundress association as a subordinate but did not lay eggs.

Including female choice led to a moderate increase in mating success (Fig. 2I). Experiment 2 had more mating attempts [odds ratio = 0.68 (95% confidence interval: 0.39–1.19), $P = 0.18$] and successful matings [odds ratio = 0.45 (95% confidence interval: 0.23–0.87), $P = 0.019$] than experiment 1. Combining the results of experiments 1 and 2, we estimate prezygotic reproductive isolation between *P. fuscatus* and *P. metricus* as 0.91.

Males from different sources (wild caught or laboratory eclosed) attempted to mate [odds ratio = 1.54 (95% confidence interval: 0.68–3.5), $P = 0.30$] and mated [odds ratio = 1.3 (95% confidence interval: 0.51–3.53), $P = 0.58$] at similar frequencies. Male body weight did not influence female mate choice in experiment 2. *Polistes fuscatus* females did not mate preferentially with the smaller male (paired t -test: $t = 1.3$, d.f. = 15.7, $P = 0.21$), nor did *P. metricus* females prefer the larger male (paired t -test: $t = -0.5$, d.f. = 13.9, $P = 0.62$). In three of the conspecific trials (3%), the female mated with both males.

In five trials (5.1%), one male attempted to mate with the other male. Male–male mating occurred in only a single trial with the same species pairing. During this trial, a *P. fuscatus* male first mated with the *P. fuscatus* female. The same male then attempted to mate with the other male. The remaining four trials with male–male mating attempts were pairings between a *P. metricus* female and *P. fuscatus* males. In all four trials, the male initially inspected, but did not mate with, the female before mounting the other male. Body weight did not predict which male mounted the other male (paired t -test: $t = -1.19$, d.f. = 5.5, $P = 0.28$).

DISCUSSION

Mate choice experiments showed strong, symmetric, prezygotic isolation between *P. fuscatus* and *P. metricus*. Male wasps were less likely to attempt to mate with heterospecifics than with conspecifics, although males attempted to mate with females of the other species in ~10% of heterospecific mating trials. Matching prior observations, males did not perform any obvious courtship behaviour towards females. As a result, female assessment of species identity and male quality probably relied on visual or olfactory cues. Male wasps had skewed mating success, with some males mating multiple times, suggesting that certain males were perceived to be of higher quality by females. Female wasps were choosy and frequently rejected undesirable males through aggressive interactions. This led to a low level of mating success even among correct species pairings, matching previous observations of mating

Experiment 1

Experiment 2

Figure 2. Quantitative measurements of male mating behaviour in experiment 1 shows that males approach (A), mount/attempt to mate (B) and mate (C) with conspecific females more quickly than with heterospecific females. Males spend more time approaching (D) and attempting to mate (E) with conspecific females during the first 30 min of each trial. Heterospecific pairings did not mate during any trial in experiment 1. (F) female *Polistes fuscatus* were slightly more aggressive than *Polistes metricus* females. Experiment 2 showed similar results, with males attempting to mate (G) and mating (H) with conspecific females more quickly than with heterospecific females. There was a small increase in the proportion of trials during which males attempted to mate and mated in experiment 2 compared with experiment 1 (I).

in *P. fuscatus* (Post & Jeanne, 1983). In these trials, successful mating was primarily determined through female choice.

Polistes fuscatus females were larger than *P. fuscatus* males and, conversely, *P. metricus* females were smaller than *P. metricus* males. Boomsma *et al.* (2005) predicted that social insects will have little sexual dimorphism, but

when sexual dimorphism exists, males should be smaller than females. The higher body weight in *P. metricus* males appears to contradict these findings. Experiment 1 suggested that *P. fuscatus* females preferred smaller males whereas *P. metricus* females preferred larger males, but this result was not significant in experiment 2. However, one possible confounding factor was our use of

Table 2. Summary of results from mate choice experiment 2

Type	Trials	Attempts	Latency to attempt	Matings	Latency to mate
<i>P. fuscatus</i> female × <i>P. fuscatus</i> male	31	17	176.9 ± 87.2	12	281.2 ± 125.4
<i>P. fuscatus</i> female × <i>P. metricus</i> male	24	2	11.7 ±	1	0.01
<i>P. metricus</i> female × <i>P. fuscatus</i> male	23	1	1142.6 ±	1	1153.9
<i>P. metricus</i> female × <i>P. metricus</i> male	21	14	186.1 ± 47.7	10	456.3 ± 182.7

Attempts and matings refer to the number of trials during which a male performed that behaviour. Latency to attempt and latency to mate are the mean and standard error of the time it took a male to perform that behaviour during the trial (in seconds).

body weight rather than body length to assess male size. Body weight may be affected by the nutritional condition; therefore, the role of male body size in mate choice is still uncertain for these species.

Prezygotic discrimination between *P. fuscatus* and *P. metricus* was not complete. In two trials, females mated with heterospecific males. Neither of these trials produced offspring, but further studies are necessary to determine whether additional post-mating isolating mechanisms exist between these species. Nevertheless, our results suggest the potential for hybridization and gene flow between *P. fuscatus* and *P. metricus* in the wild.

If mating success in these species is driven by female choice, interspecific mating is more likely to occur when females have incomplete or inaccurate information about male quality. This could occur when one species is rare and the other species common, such as at range limits. Alternatively, if females are using visual or olfactory cues to choose a mate, interspecific mating might occur in situations in which these signals are obscured, such as at crowded lekking sites containing many males of multiple species. Lastly, interspecific mating could occur when males are able to circumvent female choice, such as coercive mating between large males and small females, which might have been the case in the heterospecific matings in our study.

Poor mate choice decisions can also occur when high-quality heterospecifics are confused with conspecifics, leading to a conflict between species choice and mate choice (Pfennig, 1998). In social hymenopterans, sex is determined through single-locus complementary sex determination. When a female mates with a male with the same allele at the sex-determination locus, half of diploid offspring produced will be sterile males, leading to a severe fitness cost to the nest (Crozier, 1977). A byproduct of inbreeding avoidance could be an increase in heterospecific matings. In addition, a poor mate choice decision might be better than not mating. Mated *Bombus* gynes have higher rates of winter survival than unmated gynes (Baer & Schmid-Hempel, 2001; Gerloff & Schmid-Hempel, 2005), and there is some indication of similar benefits of mating in *P. dominula* (Izzo, 2011), suggesting that males provide unknown supplemental resources for females.

Our experiments found that male wasps had a larger margin of error for incorrect species choice. This was evidenced by male attempts to mate with other males in experiment 2. Male–male mating behaviour in insects is thought to represent a case of mistaken identity and inaccurate mate choice (Scharf & Martin, 2013; Sales *et al.*, 2018). Male–male mating attempts occurred at a similar frequency to male attempts to mate with heterospecific females.

Overall, we found strong but incomplete mate choice discrimination between *P. fuscatus* and *P. metricus*. Future studies are needed to determine which cues are important for distinguishing species identity during mate choice and to determine the frequency of hybridization between these species in nature.

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