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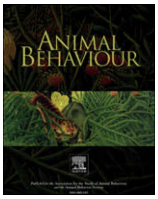

# Courtship and mate discrimination within and between species of *Timema* walking-sticks

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A R T I C L E I N F O

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The evolution of premating isolation via divergence in mating behaviour has been strongly implicated in the process of speciation. *Timema* walking-sticks show weak to moderate reproductive isolation between populations of the same species on different host plants, and high levels of isolation between species. In this paper we conducted experimental studies of within-species and between-species mating behaviour in *Timema* to address two central issues pertaining to the influence of behaviour on speciation: (1) how divergence in courtship influences reproductive isolation within and between species, and (2) whether the same or different traits mediate premating isolation within and between species. Mating behaviour involves three phases: pairing (whereby the male climbs onto the female's dorsal surface), courtship (which involves leg and antenna waving), and copulation. We found that courtship was qualitatively similar across the genus, but there were statistically significant quantitative differences in leg and antenna waving frequencies between *Timema* species. However, no-choice trials within and between species showed that discrimination within species is a function of whether males will court females after pairing, while discrimination between species is a function of whether males and females will pair. Because in both cases mate discrimination occurs before courtship is performed, we infer that courtship does not directly influence reproductive isolation in *Timema*. Moreover, because within-species and between-species discrimination take place at different stages in the mating sequence, intraspecific mate discrimination and species recognition appear to represent distinct processes. These findings suggest that, at least in *Timema*, speciation may involve the accumulation or replacement of mate discrimination mechanisms used within species.

Understanding the evolution of premating isolation is central to the study of speciation. Prezygotic isolation has been shown to evolve more quickly than postzygotic isolation in several taxonomic groups (Coyne & Orr 1989, 1997; Grant & Grant 1996, 1997), and it represents a common and effective mechanism of reproductive isolation (Gavrilets & Boake 1998). Behavioural isolation in particular has been suggested as central to the evolution of reproductive isolation (Mayr 1963; Butlin & Ritchie 1994; Coyne & Orr 2004). Among forms of behavioural isolation, courtship has been implicated in the evolution of reproductive isolation based on behaviour, because it has the potential to diverge very rapidly (Ritchie & Gleason 1995; Henry et al. 2002; Etges et al. 2006), and it effectively isolates populations or species across diverse taxa (e.g. Hoikkala & Welbergen 1995; Hoikkala et al. 2000; Tanuja et al. 2001; Henry et al. 2002).

Given the potential importance of courtship behaviour in speciation, it is crucial to investigate the dynamics of behavioural courtship signals and their effect on mating decisions and reproductive isolation across populations and species in different stages of the speciation process (Boake 2000). For example, courtship signals mediating mate choice within species may diverge sufficiently to act as behavioural prezygotic isolating mechanisms between species. This scenario has a strong theoretical basis (Lande 1981; Kirkpatrick 1982), whereby traits used to assess mate quality within species may promote a runaway process that causes populations to diverge and eventually speciate, such that sexually selected traits come to serve as cues used in species recognition (Boake et al. 1997). Such a process has been empirically supported in some groups (Hoikkala & Welbergen 1995; Blows & Allan 1998; Sadowski et al. 2002; Talyn & Dowse 2004), although the idea remains unsupported or has been refuted in other taxa (Boake et al. 1997; Carracedo et al. 2000; Hankison & Morris 2002). However, the question of whether isolating traits active in early divergence (such as between populations) are also used in species recognition has yet to be investigated.

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We examined whether similar traits underlie isolation at different stages of divergence in *Timema* walking-sticks, focusing on the role of courtship in reproductive isolation. *Timema* are a useful system for such questions because they show variable levels of reproductive isolation within and between species: for example, populations of *Timema cristinae* are adapted to different host plants (*Adenostoma* and *Ceanothus*, referred to as host types; Sandoval 1994), and show weak to moderate levels of premating isolation (Nosil et al. 2002; Nosil 2004) whereas mating is rare in no-choice trials among species (D. Arbuthnott, personal observations). Courtship behaviour has been observed informally in this group, but has yet to be characterized across species, and its importance in mate choice and species recognition have yet to be studied (Nosil et al. 2007).

In this study, we first quantified courtship for the two host types in *T. cristinae*, and for an additional nine *Timema* species. If courtship is important in reproductive isolation between host types within *T. cristinae* or between *Timema* species, then we would expect observable differences in courtship within or between species that mediate the mate discrimination process. We next performed continuously observed no-choice mate trials to determine when mating decisions are made and whether mate choice occurs before or after courtship. If mate discrimination takes place prior to courtship, then courtship is unlikely to be important in mate discrimination. We conducted no-choice mate trials both between host types in *T. cristinae* and between species using *T. cristinae*, *T. podura* and *T. chumash*. *Timema podura* and *T. chumash* were used because they show partial sympatry in nature, suggesting that species recognition may be a current focus of selection in these species. We predicted that if the same mechanisms underlie isolation at separate stages of divergence, then similar traits should be used in mating decisions regardless of the level of isolation, and mating decisions should be made at the same time for within-species and between-species mate discrimination.

## METHODS

### Insect Collection

Insects were collected from natural populations using sweep nets between March and June of 2007 and 2008. Individuals used for courtship observations were caught as adults, and males and females were housed separately. Individuals used for no-choice trials were caught as nymphs and reared to maturity with members of their native population in a 1:1 sex ratio. Although this housing arrangement results in most test individuals being nonvirgin at the time of testing, which could influence mating behaviour, housing the sexes separately greatly increases the probability of mating in a given trial (D. Arbuthnott, personal observation), making it difficult to study discrimination in these conditions. Furthermore, first-male sperm precedence does not occur in *Timema* (T. Schwander, unpublished data), so nonvirgin status probably did not influence mating behaviour on the timescale of our experiment.

### Courtship Observations

For courtship observations, one male and one female were introduced into a 6 cm petri dish in the laboratory. If the male began to court the female, we videorecorded (Panasonic PV-GS500) the subsequent behaviour of the pair, with a focus on male antennal and leg waving, the primary manifestations of courtship. All recordings were coded by D.A. using Annotation software (www.saysosoft.com, 2006).

### Intraspecific Mate Discrimination

To determine at what stage of the mating sequence mate discrimination occurs in *T. cristinae*, we conducted 505 no-choice trials (230 in 2007, 275 in 2008) using a protocol similar to that of Nosil et al. (2002). We used six populations (three of each host type) and subjected each individual to six mating trials, in which each individual was paired with an individual from one of the six populations. Sexes were separated for 3–5 h before trials. In each trial, one male and one female were introduced to a 12 cm diameter petri dish and observed continuously for 1 h. This area provided enough space for females to escape from persisting males. We recorded the occurrence and timing of the following behaviours: male–female pairing (the male climbing on the dorsal surface of the female), male courtship, rounds of courtship and copulation. To determine whether the probabilities of pairing, courtship and copulation differed between pairs of individuals from the same host plant versus different host plants, we analysed the conditional probabilities of each of these three behaviours (given the occurrence of the preceding behaviour) using logistic regression.

### Interspecific Mate Discrimination

To assess at what stage of the mating sequence mate discrimination occurs in interactions between species of *Timema*, we conducted 104 no-choice mating trials in 2008 using protocols similar to those used for intraspecific trials with *T. cristinae*. Here, all possible pairings were observed between three *Timema* species, *T. cristinae* (two populations), *T. podura* (two populations) and *T. chumash* (one population). Each individual was subjected to five mating trials, being paired with an individual from one of the five populations in each trial. Again, trials were observed for 1 h, recording pairing, courtship and mating. After an individual had been subjected to all possible mating trials, the individual was no longer used. At the end of the study, all individuals were housed with conspecifics and fed until they died naturally.

## RESULTS

### Courtship Observations

In all species, courtship was initiated by the male after he mounted the female (climbed onto her dorsal surface). Courtship involved leg waving, followed by antenna waving and copulation attempts (Fig. 1). Leg waving involved the male rapidly kicking two or four posterior legs to the side. Antenna waving involved males moving their antennae side to side, although the waving antennae rarely contacted the female. Copulation attempts followed leg and antenna waving in approximately 85% of courtship rounds, and involved the male moving his abdomen under the female's abdomen on the dextral side. Copulation attempts were observed only after courtship. When copulation attempts were unsuccessful (because females moved their abdomens and did not allow males to make genital contact), males either ceased courtship, or repeated courtship until copulation was achieved. After ceasing courtship, males remained motionless while resting on the female's dorsal surface. Characteristics of each species' courtship, including leg and antenna waving frequencies and time spent engaged in each behaviour, are provided in Table 1.

Leg waving frequencies did not differ significantly between host types of *T. cristinae* (Student *t* test: $t_{21} = -0.72$, $P = 0.48$), although differences in antenna waving frequencies approached significance ($t_{25} = -1.95$, $P = 0.06$). Between species, both leg waving frequency (ANOVA: $F_{1,9} = 2.87$, $P = 0.007$) and antenna waving frequencies ($F_{1,9} = 6.24$, $P < 0.001$) differed significantly (Table 1).

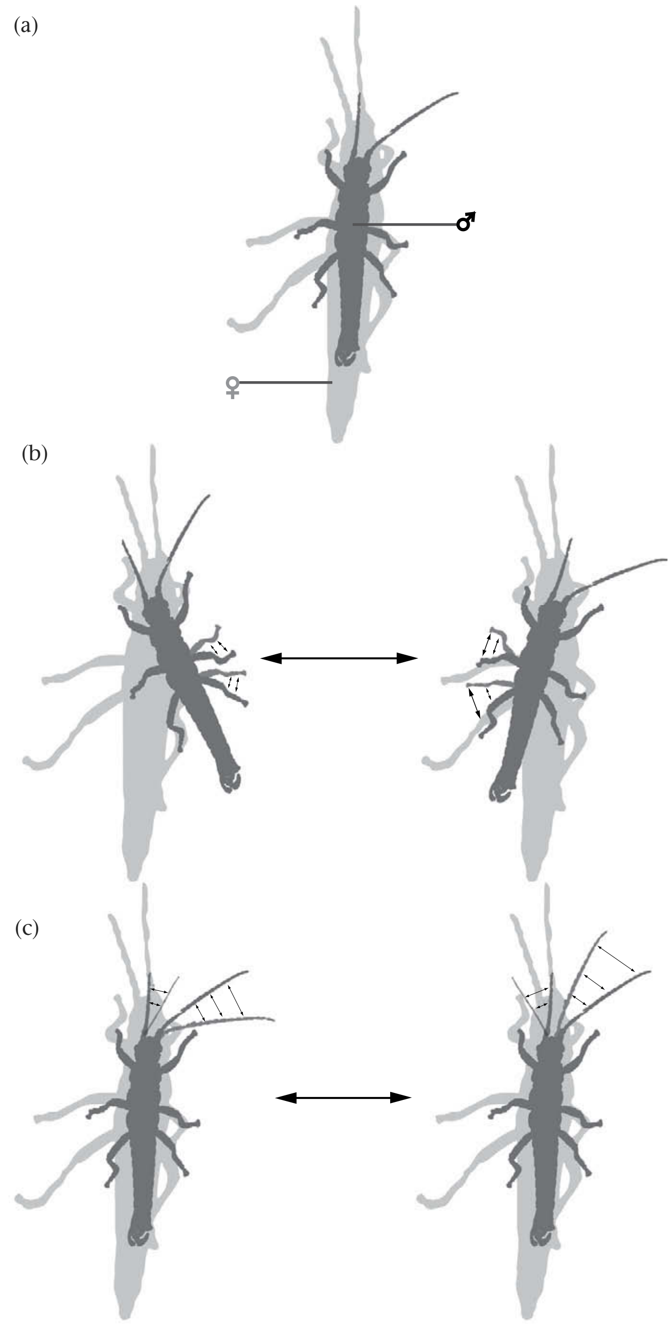

**Figure 1.** Dorsal view of typical *Timema* mating behaviour. (a) Male–female pairing: male (black) rests on female's (grey) dorsal surface. (b) Male leg waving: male kicks posterior legs from side to side. (c) Male antenna waving: male moves antennae from side to side. See text for details.

### *Intraspecific Mate Discrimination*

Of the 230 intraspecific no-choice trials performed in 2007, 61 resulted in mating. There was no difference between trials involving individuals from the same host plant versus different host plants in pairing ($Z = -1.645$, $P = 0.10$) or in mating given courtship ($Z = -0.212$, $P = 0.83$). However, there was a significant difference in courtship given pairing, such that males were more likely to court females from the same host plant ($Z = 2.236$, $P = 0.025$; Fig. 2). Across all pairings, the probability of mating decreased with increasing number of courtship bouts by males (logistic regression: $Z = -4.759$, $P < 0.001$).

In 2008, 45 of the 275 no-choice trials resulted in mating. There was no significant difference between same and different host trials for pairing ($Z = -0.814$, $P = 0.42$), courtship given pairing ($Z = -0.528$, $P = 0.60$), or mating given courtship ($Z = 1.353$, $P = 0.18$), although the absolute frequencies of courtship and mating were, for unknown reasons, lower than in 2007 (Fig. 2).

### *Interspecific Mate Discrimination*

Table 2 summarizes the results of 104 interspecific no-choice trials. Pairing was significantly more frequent in conspecific trials than in heterospecific trials ($Z = 5.25$, $P < 0.001$). Courtship given pairing did not differ significantly between conspecific and heterospecific trials ($Z = 1.608$, $P = 0.11$), although no males courted heterospecific females; this lack of significant difference can be attributed to relatively low frequencies (about 30%) of courtships given pairing for conspecifics in 2008. Because there was no heterospecific courtship, the conditional probability of mating given courtship could not be calculated for these trials.

Higher frequencies of pairing in conspecific versus heterospecific trials, with no significant differences in courtship, were consistent across all species (Fig. 3). When considering only trials where *T. cristinae* was present, pairing was more frequent between conspecifics than between heterospecifics ($Z = 2.28$, $P = 0.\ 023$), but there was no significant difference in courtship given pairing ($Z = 0.004$, $P = 0.99$). When only *T. podura* trials were considered, there was a difference in pairing ($Z = 3.91$, $P < 0.001$), but not in courtship given pairing ($Z = 0.006$, $P = 0.99$). For *T. chumash* trials, the higher frequency of conspecific pairing relative to heterospecific pairing approached significance ($Z = 1.88$, $P = 0.06$), but there was no difference in courtship ($Z = 0.003$, $P = 0.99$).

## DISCUSSION

We examined mating behaviour within and between species of *Timema* walking-sticks to characterize courtship behaviour, determine whether courtship influences either intraspecific or interspecific mate discrimination, and assess whether intraspecific and interspecific mate discrimination occur at the same stage of the mating sequence. Our primary conclusion is that both intraspecific and interspecific mate discrimination occur predominantly before males perform courtship, and at different stages in the mating sequence, which suggests that these two forms of discrimination are mediated by different mechanisms.

### *Pairing, Courtship and Mating Behaviour*

This study provides the first descriptions of *Timema* courtship behaviour. Mating behaviour and courtship were qualitatively similar across all observed *Timema* species, in that the same, simple sequence was observed. However, there were significant differences between *Timema* species, and differences in antenna waving frequency approached significance for *T. cristinae* on *Adenostoma* versus *Ceanothus* host plants. Despite the presence of differences in courtship behaviour, intraspecific no-choice mate trials showed that, between host types of *T. cristinae*, mate discrimination was manifested as decreased initiation of courtship after pairing, while in interspecific no-choice mate trials it mainly involved decreased levels of initial pairing between heterospecific males and females. There was thus no evidence of discrimination against different host types or different species after courtship was performed, in any of the no-choice mate trials. We therefore infer that courtship is not currently of primary importance in maintaining reproductive isolation, either between host types or between species of *Timema*.

**Table 1**
Courtship characteristics for *Timema*

| *Timema* species | Number of observations | Hosts* | Number of legs used in leg waving | Average leg waving frequency (SD)† | % Active courtship spent leg waving (SD)† | Average antenna waving frequency (SD)‡ | % Active courtship spent antenna waving (SD) | Notes |
|---|---|---|---|---|---|---|---|---|
| *bartmani* | 4 | F | 4 | 3.858 (1.054)$^{a,b}$ | 80 (14) | 2.16 (0.38)$^{b}$ | 20 (15) | Several short bouts of leg waving followed by antenna waving |
| *boharti* | 5 | E, F | 4 | 5.292 (2.85)$^{b}$ | 27 (14) | 1.693 (0.66)$^{a,b}$ | 73 (14) | |
| *californicum* | 5 | G | 4 | 4.15 (0.92)$^{a,b}$ | 40 (19) | 1.629 (0.24)$^{a,b}$ | 60 (19) | |
| *chumash* | 7 | D, E | 2 | 3.861 (1.19)$^{a,b}$ | 28 (18) | 2.032 (0.3)$^{b}$ | 72 (18) | |
| *cristinae* | 27 | D, E | 2 | 3.412 (1.2)$^{a,b}$ | 30 (18) | 1.465 (0.41)$^{a}$ | 70 (18) | |
| *cristinae* A | 15 | D | 2 | 3.568 (1.17)$^{a,b}$ | 31 (20) | 1.592 (0.45)$^{a,b}$ | 69 (20) | |
| *cristinae* C | 12 | E | 2 | 3.214 (1.27)$^{a,b}$ | 28 (16) | 1.306 (0.31)$^{a}$ | 72 (16) | |
| *knulli* | 5 | B | 4 | 4.578 (0.63)$^{a,b}$ | 47 (16) | 1.048 (0.19)$^{a}$ | 53 (16) | |
| *landelsensis* | 6 | C | 4 | 3.67 (1.24)$^{a,b}$ | 24 (12) | 1.566 (0.23)$^{a,b}$ | 76 (12) | |
| *petita* | 5 | D | 4 | 4.45 (0.72)$^{a,b}$ | 20 (10) | 1.23 (0.8)$^{a,b}$ | 80 (10) | |
| *podura* | 9 | D | 2 | 1.333 (1.06)$^{a}$ | 7 (15) | 2.168 (0.44)$^{b}$ | 93 (15) | Most males do not perform leg waving |
| *poppensis* | 6 | A | 4 | 2.476 (1.0)$^{a}$ | 21 (10) | 1.08 (0.2)$^{a}$ | 79 (10) | |

* Hosts are noted as in Law & Crespi (2002): A: *Pseudotsuga menziesii*; B: *Sequoia sempervirens*; C: *Arctostaphylos* species (*manzanita*); D: *Ceanothus* species; E: *Adenostoma fasiculatum*; F: *Abies concolor*; G: *Quercus* species.

† Waving frequencies were measured as number of waves per second. Superscript letters denote 95% confidence interval groupings.

‡ % Active courtship was calculated as time spent engaged in behaviour/(time spent engaged in leg waving + time spent engaged in antenna waving).

Differences in courtship signals failing to contribute to present reproductive isolation have also been reported in Galapagos finches (Grant & Grant 2002), and some species of *Drosophila* (Price & Boake 1995; Boake et al. 2000). In *Timema*, premating isolation appears to be determined by whether pairing or courtship are initiated, a pattern similar to that observed in isolation between *Drosophila silvestris* and *heteroneura* (Price & Boake 1995; Boake et al. 2000), and *Gryllus* crickets (Gray 2005). The absence of an

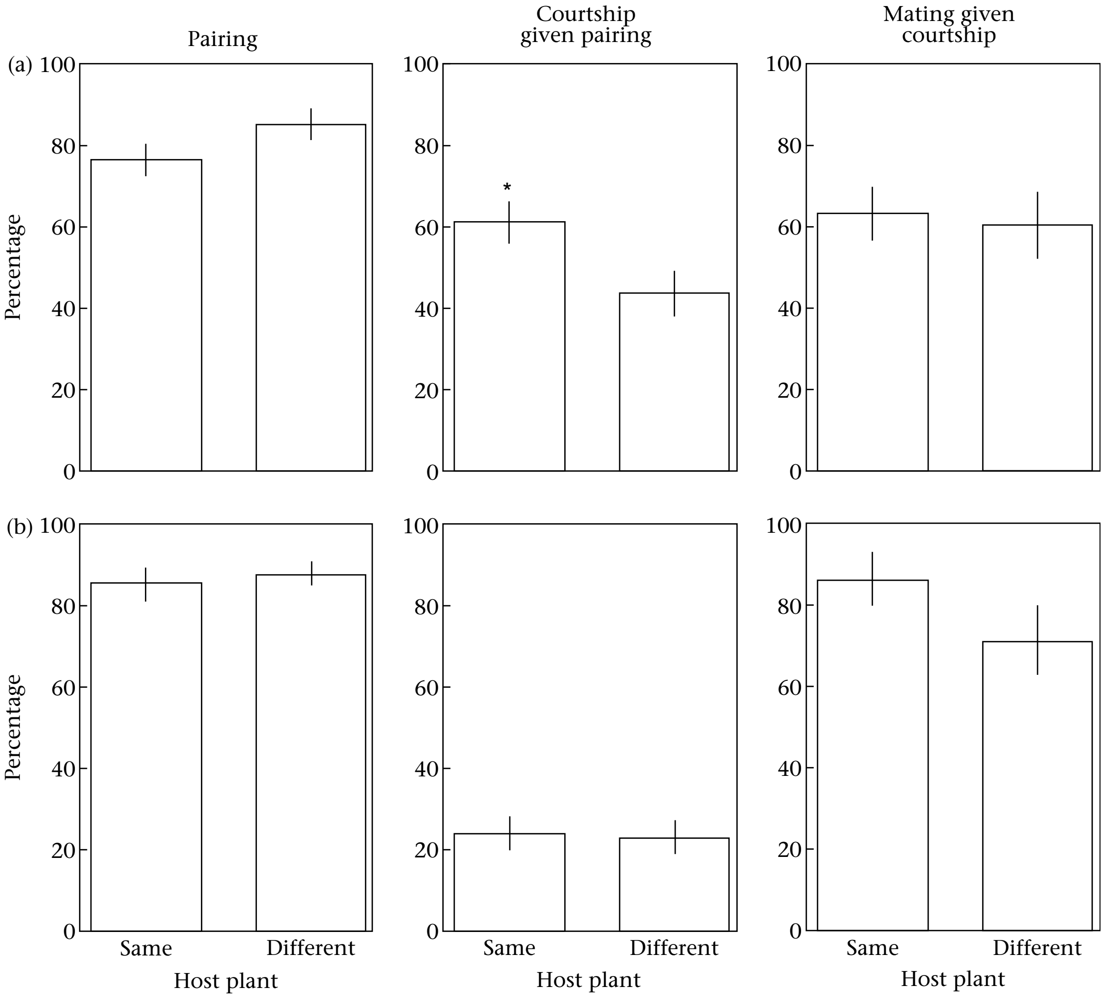

**Figure 2.** Percentage of *T. cristinae* no-choice trials that resulted in pairing, courtship given pairing and mating given courtship in (a) 2007 and (b) 2008. *$P < 0.05$.

**Table 2**
Propensity of mating behaviours for all male × female *Timema* species combinations in interspecific mate discrimination experiment

| Male | Female | Percentage of trials resulting in: | | |
|---|---|---|---|---|
| | | Pairing | Courtship given pairing | Mating given courtship |
| *cristinae* | *cristinae* | 60 | 22 | 100 |
| *cristinae* | *podura* | 16 | 0 | N/A |
| *cristinae* | *chumash* | 10 | 0 | N/A |
| *podura* | *cristinae* | 50 | 0 | N/A |
| *podura* | *podura* | 95 | 33 | 50 |
| *podura* | *chumash* | 0 | N/A | N/A |
| *chumash* | *cristinae* | 25 | 0 | N/A |
| *chumash* | *podura* | 0 | N/A | N/A |
| *chumash* | *chumash* | 40 | 50 | 0 |

obvious role for courtship in mate discrimination within or between species in these taxa raises questions regarding the current adaptive significance of courtship for males, females, or both. In *Timema*, males never attempted to copulate with a female before performing at least one bout of courtship, so we cannot draw any conclusions about its necessity in mating. However, we can suggest possibilities to be examined in future studies. First, male courtship may simply reflect a male's intentions to mate with a female. Females may require males to court to be accepted as mates, but female discrimination thresholds may be low enough that any *Timema*-like courtship is accepted. Female acceptance or rejection probably indicates a female's receptivity to mating (with any male) at the time of courtship, as there was no observable difference in acceptance or rejection rates between host types in *T. cristinae*. The finding that longer within-species courtships are relatively unlikely to result in copulation is broadly consistent with this hypothesis, in that unreceptive females will not mate with a male regardless of his mating effort. Second, courtship may represent a form of harassment, such that males increase female costs of resisting a mate (by increasing conspicuousness to predators, for example), as in waterstriders (Arnqvist & Rowe 2005). This hypothesis would help to explain why so few females behaviourally reject courting males. These two hypotheses are not mutually exclusive, and determining the function of courtship in *Timema* will require additional observations and experiments.

### *Intraspecific Mate Discrimination*

In *T. cristinae*, males and females paired indiscriminately with respect to host type, but males selectively courted females of the same host type in 2007, and there was virtually no difference in percentages of same-host plant and different-host plant matings after courtship. Because mate discrimination occurs after pairing but prior to courtship, these experiments provide evidence of a role for male mate choice in reproductive isolation between host types in *T. cristinae*, or for some male–female interaction that affects male courtship propensity. *Timema* have at least three characteristics that could promote male choice of mates (Bonduriansky 2001): long copulation times (3–5 h), during which males are unable to search for other potential mates or feed; a long period of postcopulatory mate guarding (1–4 days), which represents another significant time investment; and decreased fecundity of females mated with opposite host type individuals (Nosil 2007).

Although the methods of insect housing and no-choice trials were identical between years, there was no host-associated isolation in *T. cristinae* in 2008. The primary difference in mating patterns between 2007 and 2008 that may be associated in some way with this difference was that the absolute percentages of both courtship and mating were lower in 2008 than in 2007. Similarly, intraspecific courtship and mating frequencies were very low in the interspecific no-choice trials (which took place in 2008), which may indicate a year effect on mating propensity across *Timema* species. The reasons for this difference between years are unknown, but insects were collected one to two instars before adulthood, and were therefore subject to among-year climatic variation in the field prior to collection; in other insects, differing environmental conditions experienced as nymphs can influence levels of mate discrimination and mating propensity (Brazner & Etges 1993; Kvarnemo & Ahnesjo 1996; Engqvist & Sauer 2002). It is also possible that, because our study included many pairwise comparisons, the difference in courtship propensity within *T. cristinae* in 2007 may represent a type I error. However, this seems unlikely because host type reproductive isolation in *T. cristinae* is well documented (Nosil et al. 2002; Nosil 2004). We believe that our conclusions are not compromised by the variability in *T. cristinae* mating behaviour, as we address modes of isolation within *T. cristinae* when isolation was actually observed.

### *Interspecific Mate Discrimination*

Among *Timema* species, males and females selectively paired with conspecifics, but there was no significant difference in the conditional probabilities of courtship given pairing or mating given courtship. This selective pairing suggests that a distance or contact signal such as pheromones or cuticular hydrocarbons may be used by *Timema* to discriminate against heterospecifics. Selective pairing also suggests some role of male choice, because females resisted the pairing attempts of a heterospecific male in only 2 of 17 heterospecific pairing attempts.

Our finding that sympatric *T. podura* and *T. chumash* did not pair, although both species paired with allopatric *T. cristinae*, is consistent with a hypothesis that reinforcement of premating isolation may occur in *Timema*, as has been documented by Nosil et al. (2003) for mating patterns among populations of *T. cristinae* using different host plants. *Timema* also discriminated between species at an earlier stage (before pairing) than they did ecologically differentiated populations in *T. cristinae* (after pairing), as might be expected given higher costs of mating with heterospecifics than with conspecifics on different host plants. A hypothesis of reinforcement could be evaluated further via more extensive tests of mating patterns among and within *Timema* species in allopatry and sympatry.

### *Comparisons of Intraspecific and Interspecific Mate Discrimination*

Using time of discrimination as a proxy for traits underlying mating decisions, we infer that different traits are used for mating discrimination within and between host types in *T. cristinae*, and between species in *T. cristinae*, *T. podura* and *T. chumash*. When considering only our main focal species, *T. cristinae*, there was a clear difference in the discrimination behaviours for interacting with conspecifics versus heterospecifics, suggesting a change in the nature of isolating mechanisms during the early stages of population or species divergence, compared to after gene flow has ceased. These results suggest that changes in single traits such as contact pheromones or courtship behaviours do not both initiate divergence and isolation within species and carry this isolation through to full species status, as suggested for some systems (Lande 1981; Turner & Burrows 1995; Boake et al. 1997).

Courtship differences in particular represent effective reproductive barriers between populations and species for many taxa (e.g. Hoikkala & Welbergen 1995; Hoikkala et al. 2000; Tanuja et al. 2001; Henry et al. 2002), but the results of our study, along with those of others (e.g. Boake & Hoikkala 1995; Price & Boake 1995;

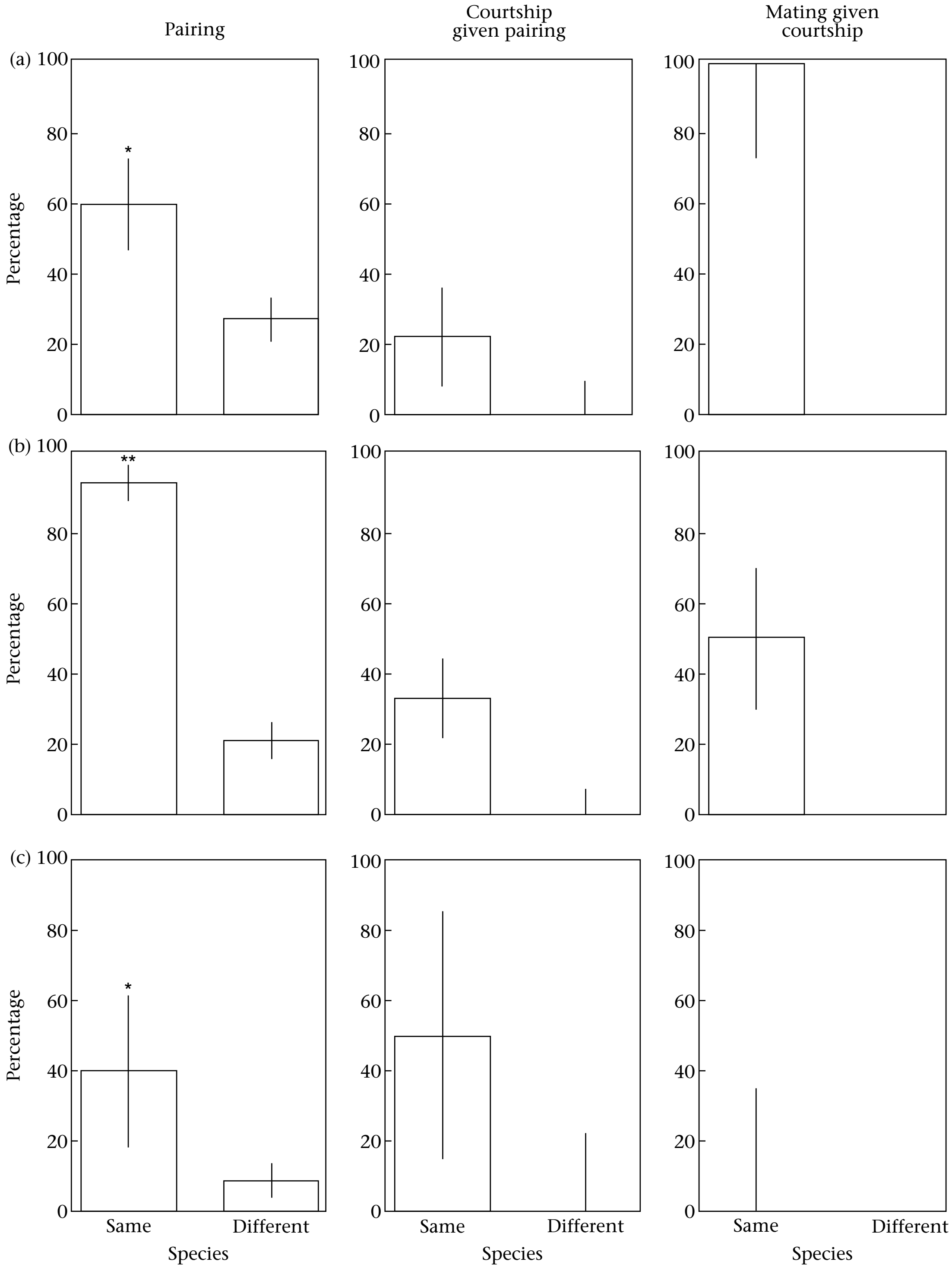

**Figure 3.** Percentage of no-choice trials that resulted in pairing, courtship given pairing and mating given courtship for (a) *T. cristinae*, (b) *T. podura* and (c) *T. chumash*. $^{*}P < 0.05$. Specific male × female pairing, courtship and mating propensities are given in Table 2.

Saarikettu et al. 2005), also show that courtship differences do not always contribute to reproductive isolation, and may therefore be relatively unimportant to speciation and species maintenance in some taxa.

Courtship is usually considered to involve predominantly female choice (Andersson 1994; Higashi et al. 1999; McPeek & Gavrilets 2006), but we found evidence consistent with male mate choice influencing mate discrimination both within and between *Timema* species, and there is increasing evidence for the importance of male choice in mate discrimination across a wide range of organisms (e.g. Bonduriansky 2001; Gowaty et al. 2003; Chenoweth & Blows 2005; Peterson et al. 2005; Bateman & Fleming 2006; Vishalakshi & Singh 2006). Male choice is predicted to be particularly important in organisms where mating engenders opportunity costs for males, as in *Timema*, where males and females remain paired for extended periods.

Continuity between mate discrimination within and between species has been supported empirically by comparing sexually selected traits with species recognition traits in some groups (Hoikkala & Welbergen 1995; Blows & Allan 1998; Sadowski et al. 2002; Talyn & Dowse 2004), but refuted in others (Boake et al. 1997; Carracedo et al. 2000; Hankison & Morris 2002). Our analyses provide a novel approach to this question, in that they involve modes of mate discrimination at both intermediate and nearly complete levels of isolation, and therefore compare early with advanced divergence. Overall, our results suggest that speciation in *Timema* involves multiple stages in which isolating mechanisms are accumulated or replaced, rather than being a continuous process.

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