COURTSHIP DIVERSIFICATION AND THE EVOLUTION OF REPRODUCTIVE ISOLATION IN *TIMEMA* WALKING-STICKS

by

Devin Arbuthnott
BSc (hons) University of Regina, 2006

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

In the
Department of Biological Sciences

SIMON FRASER UNIVERSITY

Summer 2009

APPROVAL

Name: Devin Arbuthnott

Degree: Master of Science

Title of Thesis:

Courtship diversification and the evolution of reproductive isolation in Timema walking sticks

Examining Committee:

Chair: Dr. G. Rintoul, Assistant Professor

Dr. B. Crespi, Professor, Senior Supervisor
Department of Biological Sciences, S.F.U.

Dr. F. Breden, Professor
Department of Biological Sciences, S.F.U.

Dr. B. Roitberg, Professor
Department of Biological Sciences, S.F.U.
Public Examiner

19 May 2009
Date Approved

SFU SIMON FRASER UNIVERSITY
LIBRARY

Declaration of Partial Copyright Licence

The author, whose copyright is declared on the title page of this work, has granted to Simon Fraser University the right to lend this thesis, project or extended essay to users of the Simon Fraser University Library, and to make partial or single copies only for such users or in response to a request from the library of any other university, or other educational institution, on its own behalf or for one of its users.

The author has further granted permission to Simon Fraser University to keep or make a digital copy for use in its circulating collection (currently available to the public at the "Institutional Repository" link of the SFU Library website <www.lib.sfu.ca> at: <http://ir.lib.sfu.ca/handle/1892/112>) and, without changing the content, to translate the thesis/project or extended essays, if technically possible, to any medium or format for the purpose of preservation of the digital work.

The author has further agreed that permission for multiple copying of this work for scholarly purposes may be granted by either the author or the Dean of Graduate Studies.

It is understood that copying or publication of this work for financial gain shall not be allowed without the author's written permission.

Permission for public performance, or limited permission for private scholarly use, of any multimedia materials forming part of this work, may have been granted by the author. This information may be found on the separately catalogued multimedia material and in the signed Partial Copyright Licence.

While licensing SFU to permit the above uses, the author retains copyright in the thesis, project or extended essays, including the right to change the work for subsequent purposes, including editing and publishing the work in whole or in part, and licensing other parties, as the author may desire.

The original Partial Copyright Licence attesting to these terms, and signed by this author, may be found in the original bound copy of this work, retained in the Simon Fraser University Archive.

Simon Fraser University Library
Burnaby, BC, Canada

Last revision: Spring 09

ABSTRACT

The evolution of premating isolation through changes in mating behaviour is central to the process of speciation. *Timema* walking sticks display intermediate reproductive isolation between ecologically divergent individuals within species, and almost complete isolation between species. I characterized courtship and behavioural isolation in *Timema* to examine the role of courtship in isolation, and the evolution of reproductive isolation in this genus. I found that courtship apparently plays no role in current mate discrimination within or among species, and that separate traits underlie within-species mate choice and species-recognition. However, the evolutionary history of courtship diversification implicates it in the initiation of reproductive isolation, though strengthening of isolation selects for discrimination to occur earlier as divergence progresses. Overall, I conclude that courtship currently plays little role in reproductive isolation in *Timema*, and that stages of replacement or accumulation of reproductive barriers, rather than a continuous process, characterize the evolution of reproductive isolation.

Keywords: Speciation; courtship; mating behaviour; sexual selection; species recognition; genitalic morphology

Subject Terms: Sexual selection in animals; species; courtship in animals; insects – evolution; insects – behavior; Timema

ACKNOWLEDGEMENTS

I would first like to thank Bernie Crespi for his dedicated supervision during my time at SFU. Bernie went to great lengths to provide me with advice and insights that will serve me well throughout my scientific career, and was always there to give me a nudge towards new and interesting questions. Felix Breden also provided a great deal of support and advice on my research, which helped to shape my time as a Master's student. Thanks also to Bernie Roitberg for acting as my public examiner. Tanja Schwander was and remains to be a great mentor, collaborator, and friend. Simon Goring was an extremely entertaining office mate, and somehow always knew the answer to any statistical problems I came across. Jeff Joy was always ready with scientific advice, life lessons, or inappropriate jokes, which were usually appreciated. I also owe a great deal to past and present members of the Crespi lab for useful discussions: Christine Parent, Erica Jeffery, Mick Elliot, Tanja Schwander, Jeff Joy, Lisa Raeburn, and Laura Weir. I likewise am grateful to the FAB* lab for enduring several hours of *Timema* talks and offering relevant and unique advice. Patrik Nosil provided help in the field. Mick Elliot helped with phylogenetic and Bayesion concerns. Todd Oakley kindly provided lab space in Santa Barbara. Mark McPeek and his lab have been great collaborators and I appreciate their hospitality during my stays in New Hampshire. Funding was provided by a NSERC CGS-M scholarship and a NSERC Discovery grant to Bernie Crespi.

I could not have completed this work without the constant support of my family. My parents, as well as my brothers and their partners, often calmed me down when things were going wrong in the field, and were genuinely interested in what I've been doing for the past two and a half years. Many friends at SFU have added laughter and fun to my time here, which I could not have done without. Finally, Rachel Mayberry has helped me to maintain my sanity with her love, support, humour, and random cookie baking, and has made my years here some of the best of my life.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1: INTRODUCTION

Despite a recent surge of research into speciation, the process of how species arise and diverge remains one of the least understood aspects of evolutionary biology (Coyne and Orr 2004; Hendry 2009). Two of the most significant findings of the last 20 years have been (1) the observation that prezygotic isolation can evolve faster than postzygotic isolation (Coyne and Orr 1989; Grant and Grant 1996; Coyne and Orr 1997; Grant and Grant 1997) and (2) the observation that prezygotic isolation can have a large relative contribution to total reproductive isolation (Gavrilets and Boake 1998; Coyne and Orr 2004). These observations compel a focus on the evolution of prezygotic isolation in analyses of how speciation proceeds from initiation through completion.

Prezygotic isolation can be divided into premating and postmating isolation, each with several possible mechanisms underlying isolation. Examples of premating isolating mechanisms include behavioural isolation, ecological isolation, and mechanical isolation. By contrast, examples of prezygotic-postmating isolating mechanisms include sperm competition, cryptic female choice, and gametic isolation (see Coyne and Orr 2004 for review). Changes in premating signals (such as sexual traits involved in courtship, mating, or pollination) are known to be important in the evolution of reproductive isolation in a wide range of organisms; for example, changes in floral morphology lead to pollinator isolation in *Mimulus* (Schemske and Bradshaw 1999), species-specific male genitalia lead to behavioural and mechanical isolation in *Enallagma* damselflies (McPeek et al. 2008; McPeek et al. 2009), and differences in courtship behaviour lead to behavioural isolation in the *Drosophila nasula-albomicans* complex (Tanuja et al. 2001).

Behavioural isolation has commonly been inferred as exhibiting the largest relative effect of all premating barriers on reproductive isolation (Mayr 1963; Butlin and Ritchie 1994), and mating behaviour (including female or male choice, mate recognition, and species recognition) are often the focus of such behavioural isolation (Andersson 1994). Courtship effectively isolates populations and species in some taxa (Hoikkala and Welbergen 1995; Hoikkala et al. 2000; Tanuja et al. 2001; Henry et al. 2002), which suggests that courtship can be important in the process of speciation. Understanding diversification in courtship and its impact on reproductive isolation can therefore provide valuable information on the process of speciation.

In my thesis, I have asked the following questions: 1) what are the patterns of courtship diversification? 2) Why does courtship diversify? 3) How does courtship contribute to reproductive isolation throughout the process of speciation? and 4) Is behavioural isolation within species similar to isolation between species (i.e. is the evolution of reproductive isolation continuous)? To address these questions, I compile results from relevant literature on the evolution of courtship, and combine this with empirical studies on the mating behaviour of *Timema* walking-sticks.

Timema are a wingless, phytophagous group of Phasmatodea, which are distributed throughout western United States and northern Mexico (Vickery 1993, Crespi and Sandoval 2000). There are approximately 20 known *Timema* species, which comprise 15 sexual and 5 asexual species (Vickery 1993, Law and Crespi 2002). Timema species live and feed on a range of different host plants, with some species specializing on one species of plant, while others can be found on several species (Crespi and Sandoval 2000). Host shifts within and between *Timema* species are associated with morphological divergence in order to increase crypsis on specific hosts (Nosil and Crespi 2004, Sandoval and Crespi 2008).

Timema cristinae display two genetically determined colour morphs (striped and unstriped), which represent adaptations to crypsis on their host plants, with a striped morph being more common on *Adenostoma fasciculatum* and an unstriped morph being more common on *Ceonothus spinosus* (Sandoval 1994) Individuals from different host plants can mate and are capable of producing viable offspring, but they preferentially mate with individuals from their own host plant (Nosil, 2002), and the intermediate phenotype of ‘hybrids’ have a fitness disadvantage in the form of decreased crypsis (Nosil and Crespi 2004).

Timema walking-sticks represent an excellent system to study the evolution of reproductive isolation, in that they display variable levels of isolation, which allows us to determine the mode of behavioural isolation at an incomplete level of reproductive isolation (host-types within *T. cristinae*) and subsequently compare this with the mode of behavioural isolation at a stage of complete reproductive isolation (among separate *Timema* species). Furthermore, courtship has been observed but not studied in detail in *Timema* (Nosil 2007), which allows us to examine the influence of courtship on different levels of reproductive isolation, and subsequently draw inferences concerning the importance of courtship on speciation.

References

Andersson, M. 1994. Sexual Selection. Princeton University Press, Princeton, NJ.

Butlin, R. K., and M. G. Ritchie. 1994. Behaviour and speciation. Pp. 43-79 *in* P. J. B. a. H. Slater, T. R., ed. Behaviour and Evolution. Cambridge University Press, Cambridge, UK.

Coyne, J. A., and A. H. Orr. 2004. Speciation. Sinauer Associates Publishers, Sunderland, MA.

Coyne, J. A., and H. A. Orr. 1989. Patterns of speciation in Drosophila. Evolution 43:362-381.

Coyne, J. A., and H. A. Orr. 1997. "Patterns of speciation in Drosophila" revisited. Evolution 51:295-303.

Crespi, B. J. and Sandoval, C. P. 2000. Phylogenetic evidence for the evolution of ecological specialization in *Timema* walking-sticks. Journal of Evolutionary Biology **13**: 249-262.

Gavrilets, S., and C. R. B. Boake. 1998. On the evolution of premating isolation after a founder event. Am. Nat. 152:706-716.

Grant, P. R., and B. R. Grant. 1996. Speciation and hybridization in island birds. Philos. Trans. R. Soc. Lond. Ser. B-Biol. Sci. 351:765-772.

Grant, P. R., and B. R. Grant. 1997. Genetics and the origin of bird species. Proc. Natl. Acad. Sci. U. S. A. 94:7768-7775.

Hendry, A. P. 2009. Evolutionary biology: Speciation. Nature 458:162-164.

Henry, C. S., M. L. M. Wells, and K. E. Holsinger. 2002. The inheritance of mating songs in two cryptic, sibling lacewing species (Neuroptera : Chrysopidae : Chrysoperla). Genetica 116:269-289.

Hoikkala, A., S. Crossley, and C. Castillo-Melendez. 2000. Copulatory courtship in Drosophila birchii and D. serrata, species recognition and sexual selection. J. Insect Behav. 13:361-373.

Hoikkala, A., and P. Welbergen. 1995. Signals and responses of females and males in successful and unsuccessful courtships of 3 Hawaiian lek-mating Drosophila species. Anim. Behav. 50:177-190.

Law, J. H. & Crespi, B. J. 2002. The evolution of geographic parthenogenesis in Timema walking-sticks. Mol. Ecol., 11, 1471-1489.

Mayr, E. 1963. Animal Species and Evolution. Belknap Press, Cambridge, MA.

McPeek, M. A., L. Shen, and H. Farid. 2009. The correlated evolution of three-dimensional reproductive structures between male and female damselflies. Evolution 63:73-83.

McPeek, M. A., L. Shen, J. Z. Torrey, and H. Farid. 2008. The tempo and mode of three-dimensional morphological evolution in male reproductive structures. Am. Nat. 171:E158-E178.

Nosil, P. 2007. Divergent host plant adaptation and reproductive isolation between ecotypes of *Timema cristinae* walking sticks. Am. Nat. **169**: 151-162.

Nosil, P., Crespi, B. J., and Sandoval, C. P. 2002. Host-plant adaptation drives the parallel evolution of reproductive isolation. Nature **417**: 440-443.

Nosil, P. and Crespi, B. J. 2004. Does gene flow constrain adaptive divergence or vice versa? A test using ecomorphology and sexual isolation in *Timema cristinae* walking-sticks. Evolution **58**: 102-112.

Sandoval, C. P. 1994. The effects of the relative geographic scales of gene flow and selection on morph frequencies in the walking-stick *Timema cristinae*. *Evolution* **48**: 1866-1879.

Sandoval, C. P. and Crespi, B. J. 2008. Adaptive evolution of cryptic coloration: the shape of host plants and dorsal stripes in Timema walking-sticks. Biol. J. Linn. Soc. **94**: 1-5.

Schemske, D. W., and H. D. Bradshaw. 1999. Pollinator preference and the evolution of floral traits in monkeyflowers (Mimulus). Proc. Natl. Acad. Sci. U. S. A. 96:11910-11915.

Tanuja, M. T., N. B. Ramachandra, and H. A. Ranganath. 2001. Incipient sexual isolation in the nasuta-albomicans complex of Drosophila: mating preference in male-, female- and multiple-choice mating experiments. J. Biosci. 26:365-371.

Vickery, V. R. 1993. Revision of *Timema* Scudder (Phasmatoptera: Timematodea) including three new species. Can. Ent. **125**: 657-692.

CHAPTER 2: THE GENETIC ARCHITECTURE OF INSECT COURTSHIP BEHAVIOUR AND PREMATING ISOLATION

In Press, Heredity. Accepted Dec. 2008. Final Acceptance Jan. 2009.

Abstract

The genetic architecture underlying reproductively isolating traits may have substantial impacts upon the likelihood and pace of speciation. Recent studies of a key premating barrier, courtship, provide sufficient data to assess the degree to which behaviourally isolating traits are controlled by many or few loci, and to investigate whether the same loci underlie both intraspecific and interspecific behavioural differences. Of the behavioural courtship traits examined, 69% (25 of 36) were found to be mediated by few loci of relatively large effect. This apparent prevalence of major loci suggests that changes in courtship behaviour may often evolve quickly, which in turn may drive rapid speciation through premating isolation. Although both intraspecific and interspecific courtship differences are commonly controlled by major loci, intra-specific and inter-specific differences usually involve different loci or traits. This finding provides evidence that different sets of processes and genetic changes characterize microevolutionary change in courtship-related traits, in contrast to change during speciation.

Introduction

Courtship behaviour plays a central role in isolation between many species (e.g. Hoikkala et al., 2000; Williams et al., 2001; Gleason et al., 2002; Gleason and Ritchie, 2004; Mackay et al., 2005), and the genetic architecture of such behaviour may have important implications for the process of speciation (Gavrilets and Boake, 1998; Via and Hawthorne, 1998; Henry et al., 2002; Coyne and Orr, 1989, 1997, 2004). Recent models have suggested that divergence among populations, and consequent speciation, may be more likely when traits are controlled by few loci (Arnegard and Kondrashov, 2004; Gavrilets et al., 2007; Gavrilets and Vose, 2007; Hayashi et al., 2007), and that speciation may also occur more rapidly under such genetic architecture (Gavrilets and Vose, 2007).

Previous laboratory studies have led some researchers to conclude that the interspecific differences in phenotypes that cause reproductive isolation are most commonly polygenic (e.g. Beukeboom and van den Assem, 2001; for review, see Coyne and Orr, 1998), and that mating behaviours represent complex traits controlled by many loci of small effect (Mackay et al., 2005). In the most recent review of the genetics of different mating signals that lead to reproductive isolation, Ritchie and Phillips (1998) concluded that song differences are polygenic, while few genes of major effect usually control pheromone profiles; they also proposed that quantitative differences are usually polygenic, while qualitative differences are mediated by effects of major genes. However, these authors, as well as Coyne and Orr (1998), also concluded that there were not enough data for robust cross-taxon generalizations regarding the genetics underlying premating isolation.

Since 1998, a considerable amount of data on the genetic architecture of courtship has accumulated, enough to warrant a re-evaluation of this key question. In this review I assess available data on the number and effect size of loci underlying behavioural courtship traits, mainly for traits involving auditory phenotypes in *Drosophila* and other insects. I also assess whether the same or different loci and traits underlie species recognition and intraspecific mate choice, to help determine if the processes involved in intraspecific mate choice also influence the evolution of reproductive isolation (Lande 1981; Turner and Burrows, 1995; Boake et al., 1997; Blows and Higgie, 2002; Gleason and Ritchie 2004).

Methods

Selection of traits

Courtship can be treated as a single phenotypic trait or a combination of several individual characters (Etges et al., 2006). To draw meaningful conclusions about reproductive isolation, one must reliably identify which particular traits are acting as reproductive barriers (Via and Hawthorne, 1998; Etges et al., 2007); determining that a character is species specific is not equivalent to determining its role in isolation. Some researchers have measured the courtship phenotype as a whole by quantifying attractiveness to the opposite sex (e.g. Henry et al., 2002; Moehring et al., 2004; Klappert et al., 2007). Anholt and Mackay (2004) suggest that one can quantify courtship by simply measuring latency to courtship and copulation. Although such measures are useful in quantifying premating isolation as a whole, they integrate a large collection of traits, including male activity, mate recognition, male choice, female resistance, female choice, and the complexity of courtship, making these measures relatively uninformative about what specific traits underlie isolation. Moreover, as pointed out by Boake et al. (1998), because such summary traits likely represent several characters, they are predisposed to reveal a polygenic architecture, biasing studies of the genetics of reproductive isolation towards detection of such effects. In this review I thus concentrate on the genetic bases of the specific phenotypic traits involved in courtship and reproductive isolation.

Major loci versus polygenic effects

Major-locus and polygenic effects represent ends of a continuum (Falconer and Mackay, 1996), and authors vary in their criteria for concluding either simple or complex genetic architecture. Some studies make a distinction between major-locus and polygenic distinction by determining the percent of phenotypic variance explained by target loci (e.g. Hoikkala et al., 2000; Gleason et al., 2002); by contrast other studies base their conclusions on the number of loci underlying a trait relative to genome size, or the distribution of loci throughout the genome (e.g. Yeh et al., 2006; Klappert et al., 2007). In this review I describe the criteria used by different authors for such conclusions alongside the results, and present both the conclusions of the authors and the percent of phenotypic variance explained.

Regardless of the criteria used to determine simple versus complex genetic architecture, most genetic methods show a bias towards detecting loci of large effect, as with QTL studies (Via and Hawthorne, 1998). Furthermore, Orr's (1998) theoretical models predict that we should find a mixed (exponential) distribution of genetic effect sizes, meaning that there should be few loci of large effect, many loci of intermediate effect, and many more loci of small effect. Given that genetic methods are limited in their ability to detect loci of small effect, there is an expected bias to the detection of loci of large and intermediate effect, while the many loci of small effect may go undetected. It is therefore important to consider these methodological biases when interpreting the results of the available data on the genetic architecture of courtship.

Literature surveyed

This review focuses on courtship-related signals, mainly in auditory traits in *Drosophila*, which represents the primary source of information in the literature. As genetic analyses of courtship extend to non-insects, and a diversity of traits, the generality of the patterns inferred here can be evaluated. The term 'genetic architecture' refers here the number and locations of loci underlying courtship traits. This scale of analysis allows conclusions to be drawn regarding genomic regions, rather than specific genes; such regions may vary considerably in size (Paterson et al., 1988), and a locus originally recognized initially to be of large effect may actually comprise several genes of relatively-small effect (e. g., Perez et al., 1993; Perez and Wu, 1995). However, the literature pertaining to genetic architecture is dominated by analysis of loci, making this the optimal level of analysis in which to make conclusions on the evolutionary dynamics of courtship traits and premating isolation.

To be included in this review, a study had to either (i) make an inference about the genetic architecture of a behaviour involved in courtship, or (ii) provide an estimated number of loci involved in a courtship behaviour. A list of related references which were not included in this review are provided in the Appendix.

Results

How many loci control courtship differences?

The results from 20 studies, representing 33 characters and 24 species, are summarized in Table 2-1. Of the traits in which a genetic architecture was inferred by the authors (authors' criteria for these observations are summarized in column 7 of Table 2-1), 25 of 36 (69%) were considered to be influenced by few loci of large effect. Percent of phenotypic variance explained by one locus ranged from 9 to 40% (average 23%) in those studies that present this information. Of those traits concluded to be under simple genetic control, the variance explained ranged from 19% to 40% (average 30%), while those traits concluded to be polygenic ranged from 9% to 14% (average 10%).

Some data that did not meet all of the criteria to be included in Table 2-1 (because they measured isolation as a whole rather than a behaviour, did not make an inference regarding genetic architecture, or did not present the number of loci) are also relevant to this compilation. Few loci (2 or 3, depending on the backcross used) of large effect were found to be responsible for sexual isolation, measured as copulation occurrence, in *Drosophila yakuba* and *D. santomea* (Moehring et al., 2006). Similarly, Noor et al. (2001) concluded that sexual isolation (through courtship dysfunction of hybrids) between *Drosophila pseudoobscura* and *D. persimilis* was mediated by three loci of large effect. By contrast, Jia et al. (2000) found that many loci control sexually selected characters (components of male ultrasonic signals) in the wax moth *Achroia grisella*, and Mackay et al. (2005) have suggested that up to 21% of the genome may be involved in the mating speed of *Drosophila*. Considered together with the studies in Table 2-1, this evaluation of the genetic basis of traits involved in pre-mating isolation indicates that large effects of few loci underlie variation in over two-thirds of the traits examined.

In some species pairs (*D. ananassae/ pallidosa*, *Chrysoperla plorabunda/johnsoni*, *Chorothippus albomarginatus/oschei*, *Chorothippus brunneus/jacobsi*, and *Laupala paranigra/kohalensis*), reproductive isolation has been considered to evolve quickly through changes in behaviour alone, as there is a lack of postzygotic isolation, and ecology and morphology may be identical between species (Henry et al., 2002; Yamada et al., 2002; Saldamando et al., 2005; Shaw et al., 2007; Vedenina et al., 2007). When this subset of species pairs for which behavioural isolation represents the only reproductive barrier, or where speciation is known to be rapid, is considered, a similar proportion, five of the nine traits (56%), were controlled by major loci, while the other four were polygenic.

Intra- versus interspecific changes in courtship

From Table 2-1, nine of 13 differences (69%) within species are mediated by few loci of large effect, whereas 16 of 23 differences (70%) between species are through few loci of large effect. The majority of both intraspecific and interspecific differences in courtship traits are thus mediated by few loci of large effect. Similar genetic architecture underlying the two comparisons does not, however, mean that the same mechanisms underlie each. In fact, the majority of studies that discuss intra-specific versus inter-specific differences conclude that they do not involve the same traits. For example, intraspecific differences in the interpulse intervals (IPI) of *Drosophila melanogaster* are underlain by few loci of major effect, while interspecific differences in IPI of *Drosophila simulans* and *D. sechellia* have apparently evolved via many loci of small effect, which indicates that sexual selection and species recognition do not represent a continuum in these species (Gleason and Ritchie, 2004). A similar pattern is seen for species in the *Drosophila virilis* and *D. montana* groups, as well as the observation that intraspecific differences involve autosomes, whereas interspecific differences are largely influenced by the X chromosome (Hoikkala et al., 2005). Lastly, Carracedo et al. (2000) determined that *Drosophila melanogaster*, *D. simulans*, and *D. mauritiana* all use different discrimination mechanisms when interacting with heteropecifics versus conspecifics, and that interspecific genetic differences in sexually isolating traits originate after speciation.

Many additional studies have found that the loci responsible for intraspecific differences are located on different chromosomal arms or different chromosomes altogether than loci responsible for interspecific differences in courtship behaviour (Table 2-2). When analyzed at the behavioural level, Boake et al. (1997) concluded that sexual selection and species recognition do not represent a continuum in the species pair *Drosophila silvestris* and *D. heteroneura*. Therefore, although similar genetic patterns may underlie both intraspecific mate choice and species recognition, most evidence suggests that the two processes are not driven by the same traits.

Discussion

Behavioural signaling, which plays a key role in courtship and premating isolation, has previously been thought to be mediated predominantly by many loci of small effect (e.g. Coyne and Orr, 1998; Beukeboom and van den Assem, 2001; Mackay et al. 2005). The primary conclusion of this review is that the majority of traits influencing premating isolation measured to date show a genetic architecture of few loci of major effect. Furthermore, the percentage of phenotypic variance explained is often large enough for natural or sexual selection to act on via change in a single locus. However, this pattern of mating signals controlled by few loci is far from universal, and in no way can it be considered a rule, as approximately 30% of traits are found to be under polygenic control.

One important implication of a pattern of major loci underpinning the genetic basis of courtship is that several recent models have suggested speciation may be more likely (Arnegard and Kondrashov, 2004; Gavrilets et al., 2007; Gavrilets and Vose, 2007; Hayashi et al., 2007), and divergence faster

(Gavrilets and Vose, 2007), when adaptive or reproductive traits are underlain by such simple genetic architectures. For example, in simulations by Gavrilets and Vose (2007), ecological traits controlled by four loci promoted speciation more often, and faster, than simulations where traits were controlled by eight or 16 loci, even when all selective and ecological forces were kept constant. Therefore, this study's results suggest that changes in courtship behaviour and premating isolation may often evolve quickly, which in turn may contribute to the importance of behaviour early in the speciation process. Such theoretical predictions are consistent with observations of rapid courtship divergence among some insect lineages (Ritchie and Gleason, 1995; Etges et al. 2006), although robust tests of the assumptions and predictions of such models require additional studies. A possible further test of the role of genetic architecture in speciation would involve comparing traits causing reproductive isolation with otherwise-comparable behavioural traits that do not influence isolation, to determine if isolation-related traits are more-commonly controlled by few major loci.

Henry et al. (2002) suggested that changes through few loci (which are capable of relatively-large leaps in phenotype) should be characteristic of differences predominantly in sexually-selected traits that are arbitrary with respect to natural selection, but changes through many loci (which are generally more gradual) may indicate that environmental adaptation accompanies sexual selection, making speciation adaptive, as in conditions underlying ecological speciation (Schluter, 2001; Rundle and Nosil, 2005). The data reviewed here, however, indicate that some species pairs that have diverged recently, where behaviour represents the only barrier to reproduction, exhibit polygenic architecture for isolating traits (e.g. Saldamando et al., 2005; Shaw et al., 2007; Vedenina et al., 2007), which runs counter to the predictions of Henry's (2002) hypothesis. Additional data are needed, however, for quantitative evaluation of the degree to which ecological versus non-ecological speciation are underlain by different patterns of genetic change.

Interpretation of the results presented here regarding the genetic architecture of premating isolation is subject to several important caveats. First, the apparent prevalence of major loci may, in part, reflect methodological biases, given the precision of genetic analyses and their power to detect loci of small effects; for example, some QTL methods can exhibit biases towards detection of loci of large effect (Via and Hawthorne, 1998). However, there is no reason to expect systematic biases specific to courtship-related traits. Furthermore, the studies in Table 2-1 show no evidence of association between genetic architecture and ascertainment method: 68% of traits studied through crossing and breeding experiments are controlled by major loci, while 64% of QTL analyses showed traits to be controlled by major loci.

Second, the choice of traits to examine may also influence the nature of genetic inferences, as courtship often comprises multiple behavioural components. For example, when considered a single phenotype, courtship differences between *Drosophila elegans* and *D. gunungcola* are polygenic (Yeh et al., 2006), but when each component of courtship is considered separately, they are each apparently under the control of a few major loci (Table 2-1), with wing display, body shaking, and circling mediated by effects of loci on different chromosomes. Such results demonstrate the importance of determining which traits underlie reproductive isolation.

Third, courtship need not mediate sexual selection within species, or reproductive isolation among them (e.g. Boake and Hoikkala 1995, Boake and Poulsen 1997, Price and Boake 1995, Saarikettu et al. 2005). However, the majority of traits in Table 2-1 have been determined to be important in mate choice. When considering only those traits with demonstrated direct relevance to mate choice, 14 of 19 (74%) traits are underlain by major loci. Therefore, this examination of genetic architecture of courtship is pertinent to our understanding of the evolution of reproductive isolation.

Finally, intraspecific versus interspecific locus comparisons sometimes involve intraspecific signals of *Drosophila melanogaster*, contrasted with interspecific processes as measured in separate *Drosophila* species pairs. To conclude whether the same or different traits underlie intraspecific mate choice and interspecific reproductive isolation at the genetic level, it is necessary to identify the loci influencing sexually selected traits in one species, and determine whether there is overlap with loci causing reproductive isolation between this focal species and a sister species.

A second primary conclusion of this review is that studies of both intra-specific and inter-specific differences in behavioural signaling show a prevalence of major-locus effects on variation, but that the loci involved seldom overlap. These results suggest that sexual selection and species recognition are not mediated through the same loci and traits, which implies that these two processes do not represent two ends of a continuum, at least at the level of genetic architecture. This inference does not imply that sexual selection does not drive processes of speciation in these organisms; conclusions as to the mode of speciation cannot be drawn simply from looking at the genes underlying isolation. What these data do suggest is that the same loci and traits do not underlie isolation at both the within- and between-species levels. Speciation may thus represent less a continuous process where one isolating mechanism initiates and completes reproductive isolation, than a process involving the accumulation, or replacement, of novel isolating mechanisms and traits during and after the speciation process (Boake et al. 1997; Carracedo et al. 2000).

Evidence on the inter-relatedness of sexual selection and speciation remains sparse, and perhaps insufficient to draw any general conclusions. Indeed, Price (2002) points out that "the genetics of species differences is not the genetics of speciation," given that species differences likely accumulate after speciation and processes causing isolation now may not have caused isolation during speciation. Comparative studies that integrate behavioural data on courtship and pre-zygotic isolation with information on genomic architecture, for specific clades with species at variable stages of divergence, may be most useful for assessing the genetic bases of the speciation process.

Overall, there is a prevalence of major loci in the available data for both intra- and interspecific differences in behavioural mate signaling, though approximately 30% of the traits included are under polygenic control. This data complements two other important conclusions from previous work. First, premating isolation often evolves quickly (e.g. Coyne and Orr, 1989, 1997; Grant and Grant, 1997), making it important in the early stages of speciation. Second, some recent models predict that speciation is more probable and progresses more quickly when isolating traits are through few loci (Gavrilets et al., 2007; Gavrilets and Vose, 2007; Hayashi et al., 2007). The empirical evidence presented here for the genetic control of courtship is thus generally concordant with previous empirical and theoretical work on the patterns of isolation and speciation. Courtship is often controlled by few loci, which may predispose it to fast divergence, which in turn may drive rapid speciation through premating isolation.

Acknowledgements

Thanks to Felix Breden for introducing me to questions about genetic architecture and evolution. B. J. Crespi and F. Breden provided extensive feedback and revisions for this manuscript. This work was funded by a NSERC CGS-M scholarship to D.A. and a NSERC Discovery Grant to B.J. Crespi.

Supplementary information is available at Heredity’s website.

References

Anholt RRH, Mackay TFC (2004). Quantitative genetic analyses of complex behaviours in *Drosophila*. *Nature Rev. Genet.* **5**: 838-849.

Arnegard ME, Kondrashov AS (2004). Sympatric speciation by sexual selection alone is unlikely. *Evolution* **58**: 222-237.

Beukeboom LW, van den Assem J (2001). Courtship and mating behaviour of interspecific *Nasonia* hybrids (Hymenoptera, Pteromalidae): a grandfather effect. *Behav. Genet.* **31**: 167-177.

Blows MW, Higgie M (2002). Evolutionary experiments on mate recognition in the *Drosophila serrata* species complex. *Genetica* **116**: 239-250.

Boake CRB, Hoikkala A (1995). Courtship behaviour and mating success of wild-caught *Drosophila-silvestris* males. *Anim. Behav.* **49**: 1303-1313.

Boake CRB, Poulsen T (1997). Correlates versus predictors of courtshp success: courtship song in *Drosophila silvestris* and *D- heteroneura*. *Anim. Behav.* **54**: 699-704.

Boake CRB, DeAngelis MP, Andreadis DK (1997). Is sexual selection and species recognition a continuum? Mating behaviour of the stalk-eyed fly *Drosophila heteroneura*. *Proc. Natl Acad. Sci. USA* **94**: 12442-12445.

Boake CRB, Price DK, Andreadis DK (1998). Inheritance of behavioural differences between two interfertile, sympatric species, *Drosophila silvestris* and *D. heteroneura*. *Heredity* **80**: 642-650.

Butlin RK (1996). Co-ordination of the sexual signaling system and the genetic basis of differentiation between populations in the brown planthopper, *Nilaparvata lugens*. *Heredity* **77**: 369-377.

Carracedo MC, Suarez C, Casares P (2000). Sexual isolation between *Drosophila melanogaster, D. simulans* and *D. mauritiana*: sex and species specific discrimination. *Genetica* **108**: 155-162.

Colegrave N, Hollocher H, Hinton K, Ritchie MG (2000). The courtship song of African *Drosophila melanogaster*. *J. Evol. Biol.* **13**: 143-150.

Coyne JA, Orr HA (1989). Patterns of speciation in Drosophila. *Evolution* **43**: 362-381.

Coyne JA, Orr HA (1997). “Patterns of speciation in Drosophila” revisited. *Evolution* **51**: 295-303.

Coyne JA, Orr HA (1998). The evolutionary genetics of speciation. *Phil. Trans. R. Soc. Lond. B* **353**: 287-305.

Coyne JA, Orr H A (2004). *Speciation*. Sinauer Associates Publishers. Sunderland, MA.

Etges WJ, Over KF, De Oliveira C, Ritchie MG (2006). Inheritance of courtship song variation among geographically isolated populations of *Drosophila mojavensis*. *Anim. Behav.* **71**: 1205-1214.

Etges WJ, de Oliveira CC, Gragg E, Oritz-Barrientos D, Noor MAF, Ritchie MG (2007). Genetics of incipient speciation in *Drosophila mojavensis* I. male courtship song, mating success, and genotype X environment interactions. *Evolution* **61**: 1106-119.

Falconer DS, Mackay TFC (1996). *Introduction to Quantitative Genetics*. Pearson Education Limited. Essex, UK.

Gavrilets S, Boake CRB (1998). On the evolution of premating isolation after a founder event. *Am. Nat.* **152**: 706-717.

Gavrilets S, Vose A, Barluenga M, Salzburger W, Meyers A (2007). Case studies and mathematical models of ecological speciation. 1. Cichlids in a crater lake. *Mol. Ecol.* **16**: 2893-2909.

Gavrilets S, Vose A (2007). Case studies and mathematical models of ecological speciation. 2. Palms on an oceanic island. *Mol. Ecol.* **16**: 2910-2921.

Gleason JM, Nuzhdin SV, Ritchie MG (2002). Quantitative trait loci affecting a courtship signal in *Drosophila melanogaster*. *Heredity* **69**: 1-6.

Gleason JM, Ritchie MG (2004). Do quantitative trait loci (QTL) for a courtship song difference between *Drosophila simulans* and *D. sechellia* coincide with candidate genes and intraspecific QTL? *Genetics* **166**: 1303-1311.

Grant PR, Grant RB (1997). Genetics and the origin of bird species. *Proc. Natl Acad. Sci. USA* **94**: 7768-7765.

Hayashi TI, Vose M, Gavrilets S (2007). Genetic differentiation by sexual conflict. *Evolution* **61**: 516-529.

Henry CS, Wells MLM, Holsinger KE (2002). The inheritance of mating songs in two cryptic, sibling lacewing species (Neuroptera: Chrysopidae: *Chrysoperla*). *Genetica* **116**: 269-289.

Hoikkala A, Päällysaho S, Aspi J, Lummer J (2000). Localization of genes affecting species differences in male courtship song between *Drosophila virilis* and *D. littoralis*. *Genet. Res. Camb.* **75**: 37-45.

Hoikkala H, Klappert K, Mazzi D (2005). Factors affecting male song evolution in *Drosophila Montana*. *Curr. Top. Dev. Biol.* **67**: 225-250.

Huttunen S, Aspi J (2003). Complex inheritance of male courtship song characters in *Drosophila virilis*. *Behav. Genet.* **33**: 17-24.

Huttunen S, Aspi J, Hoikkala A, Schlötterer C (2004). QTL analysis of variation in male courtship song characters in *Drosophila virilis*. *Heredity* **92**: 263-269.

Jia F, Greenfield MD, Collins RD (2000). Genetic variance of sexually selected traits in waxmoths: maintenance by genotype X environment interaction. *Evolution* **54**: 953-967.

Klappert K, Butlin RK, Reinhold K (2007). The attractiveness fragment- AFLP analysis of local adaptation and sexual selection in a caeliferan grasshopper *Chorthippus biguttulus*. *Naturwissenschaften* **94**: 667-674.

Lande R (1981). Models of speciation by sexual selection on polygenic traits. *Proc. Natl Acad. Sci. USA* **78**: 3721-3725.

Mackay TFC, Heinsohn SL, Lyman RF, Moehring AJ, Morgan TJ, Rollmann SM (2005). Genetics and genomics of *Drosophila* mating behaviour. *Proc. Natl Acad. Sci. USA* **102**: 6622-6629.

Moehring AJ, Li J, Schug MD, Smith SG, deAngelis M, Mackay TFC, et al. (2004). Quantitative trait loci for sexual isolation between *Drosophila simulans* and *D. mauritiana*. *Genetics* **167**: 1263-1247.

Moehring AJ, Llopart A, Elwyn S, Coyne JA, Mackay TFC (2006). The genetic basis of prezygotic reproductive isolation between *Drosophila santomea* and *C. yakuba* due to mating preference. *Genetics* **173**: 215-225.

Noor MAF, Grams KL, Bertucci LA, Almendarez Y, Reiland J, Smith KR (2001). The genetics of reproductive isolation and the potential for gene exchange between *Drosophila pseudoobscura* and *D. persimilis* via backcross hybrid males. *Evolution* **55**: 512-521.

Orr HA (1998). The population genetics of adaptation: the distribution of factors fixed during adaptive evolution. *Evolution* **52**: 935-949.

Paterson AH, Landers ES, Hewitt JD, Peterson S, Lincoln SE, Tanksley SD (1988). Resolutioon of quantitative traits into mendelian factors by using a complete linkage map of restriction fragment length polymorphisms. *Nature* **335**: 721-726.

Perez DE, Wu CI, Johnson NA, Wu ML (1993). Genetics of reproductive isolation in the *Drosophila-simulans* clade- DNA marker assisted mapping and characterization of a hybrid-male sterility gene, odysseus (ods). *Genetics* **134**: 261-275.

Perez DE, Wu CI (1995). Further characterization of the odysseus locus of hybrid sterility in *Drosophila*- one gene is not enough. *Genetics* **140**: 201-206.

Price DK, Boake CRB (1995). Behavioural reproductive isolationin *Drosophila-silvestris*, *D-heteroneura*, and their F1 hybrids (*Diptera*, *Drosophilidae*). *J. Insect Behav.* **8**: 595-616.

Price TD (2002). Domesticated birds as a model for the genetics of speciation by sexual selection. *Genetica* **116**: 311-327.

Pugh ARG, Ritchie MG (1996). Polygenic control of a mating signal in *Drosophila*. *Heredity* **77**: 378-382.

Ritchie MG, Gleason JM (1995). Rapid evolution of courtship song pattern in *Drosophila willistoni* sibling species. *J. Evol. Biol.* **8**: 463-479.

Ritchie MG, Phillips SDF (1998). The genetics of sexual isolation. In: Howard DJ, Berlocher SH (eds) *Endless Forms: Species and Speciation*, pp. 291-308. Oxford University Press, New York, NY.

Rundle HD, Nosil P (2005). Ecological speciation. *Ecol Lett* **8**: 336-352.

Saarikettu M, Liimatainen JO, Hoikkala A (2005). Intraspecific variation in mating behaviour does not cause sexual isolation between *Drosophila virilis* strains. *Anim. Behav.* **70**: 417-426.

Saldamando CI, Miyaguchi S, Tatsuta H, Kishino H, Bridle JR, Butlin RK (2005). Inheritance of song and stridulatory peg number divergence between *Chorthippus brunneus* and *C. jacobsi*, two naturally hybridizing grasshopper species (Orthoptera: Acrididae). *J. Evol. Biol.* **19**: 703-712.

Schluter D (2001). Ecology and the origin of species. *Trends Ecol. Evol.* **16**: 372-380.

Shaw KL, Parsons YM, Lesnick SC (2007). QTL analysis of a rapidly evolving speciation phenotype in the Hawaiian cricket *Laupala*. *Mol. Ecol.* **16**: 2879-2892.

Tinghitella RM (2008). Rapid evolutionary change in a sexual signal: genetic control of the mutation 'flatwing' that renders male field crickets (*Teleogryllus oceanicus*) mute. *Heredity* **100**: 261-267.

Turner GF, Burrows MT (1995). A model of sympatric speciation by sexual selection. *Proc. R. Soc. Lond. B* **260**: 287-292.

Vedenina VY, Panyutin AK, Helversen OV (2007). The unusual inheritance pattern of the courtship songs in closely related grasshopper species of the *Chorthippus albomarginatus*- group (Orthoptera: Gomphocerinae). *J. Evol. Biol.* **20**: 260-277.

Via S, Hawthorne DJ (1998). The genetics of speciation: promises and prospects of quantitative trait locus mapping. In: Howard DJ, Berlocher SH (eds) *Endless Forms: Species and Speciation*, pp. 352-364. Oxford University Press, New York, NY.

Williams MA, Blouin AG, Noor AF (2001). Courtship songs of *Drosophila pseudoobscura* and *D. persimilis*. II. Genetics of species differences. *Heredity* **86**: 68-77.

Yamada H, Matsuda M, Oguma Y (2002). Genetics of sexual isolation based on courtship song between two sympatric species: *Drosophila ananassae* and *D. pallidosa*. *Genetica* **116**: 225-237.

Yeh SD, Liou SR, True JR (2006). Genetics of divergence in male wing pigmentation and courtship behaviour between *Drosophila elegans* and *D. gunungcola*. *Heredity* **96**: 383-395.

Table 2-1: Genetic control of traits involved in courtship behaviour

Taxon and Species	**Trait**	**Selected during mate choice?**[1]	**Method**	**Loci number**	**Max effect**[2]	**polygennic or major loci**[3]	**Reference**
Drosophila							
melanogaster	Interpulse interval	Y	Chromosome replacement			Major loci[b]	Colegrave et al. 2000
	Interpulse interval	Y	QTL	3	24.9	Major loci[a]	Gleason et al. 2002
mojavensis	Interpulse interval	Y	Breeding experiment			Polygenic[b]	Etges et al. 2006
	Long Interpulse interval	Y	QTL	3			Etges et al. 2007
	Short Interpulse interval	N	QTL	1			Etges et al. 2007
	Burst duration	? (Y for D. arizonae)	Breeding experiment			Polygenic[b]	Etges et al. 2006
		Y	QTL	3			Etges et al. 2007
virilis	Pulse number	?	QTL	13	13.8	Polygenic[a,b]	Huttunen et al. 2004

	Pulse train length	?	QTL	8	9	Polygenic[a,b]	Huttunen et al. 2004
elegans/ gunungcola	Male courtship	?	Crosses + genotyping	“many”		Polygenic[b]	Yeh et al. 2006
	Wing display	?	Crosses + genotyping	“few”		Major loci[b]	Yeh et al. 2006
	Body shaking	?	Crosses + genotyping	“few”		Major loci[b]	Yeh et al. 2006
	Circling	?	Crosses + genotyping	“few”		Major loci[b]	Yeh et al. 2006
ananassae/ pallidosa	Song characters chosen by females	Y	Chromosome substitution crosses			Major loci[b]	Yamada et al. 2002
pseudoobscura/ persimilis	Interpulse interval	Y	msat, RFLP	≥3	19	Major loci[a,b]	Williams et al. 2001
	Intrapulse frequency	Y	msat, RFLP	≥2	24	Major loci[a,b]	Williams et al. 2001
simulans/ sechellia	Interpulse interval	Y	QTL	6	9.4	Polygenic[a,b]	Gleason and Ritchie 2004
simulans/ mauritiana	Male traits being selected by females	Y	QTL	3		Major loci[b]	Moehring et al. 2004

	Interpulse interval	Y	Breeding experiment and chromosome markers			Polygenic[b]	Pugh and Ritchie 1996
virilis/ littoralis	Pulse length	?	Breeding experiment and chromosome markers	5	39	Major loci[a,b]	Hoikkala et al. 2000
	Pulse train length	?	Breeding experiment and chromosome markers	2	30.6	Major loci[a,b]	Hoikkala et al. 2000
	Song pause	?	Breeding experiment and chromosome markers	6	40.4	Major loci[a,b]	Hoikkala et al. 2000
	Pulse number	?	Breeding experiment and chromosome markers	3	21.7	Major loci[a,b]	Hoikkala et al. 2000
	Cycle number	?	Breeding experiment and chromosome markers	4	36.6	Major loci[a,b]	Hoikkala et al. 2000
Neuroptera							

Chrysoperla plorabunda/ johnsoni	1st principal component of selected song traits	Y	Breeding experiment	2 – 7		Major loci[b]	Henry et al. 2002
Orthoptera							
Teleogryllus oceanicus	Presence/absence of courtship song	Y	Breeding experiment	1		Major loci[b]	Tinghitella 2008
Chorthippus biguttulus	Male song attractiveness	Y	AFLP	2		Major loci[b]	Klappert et al. 2007
C.albomarginatus/ oschei	Song amplitude	? (Y for song as a whole)	Breeding experiment	“many”		Polygenic[b]	Vedenina et al. 2007
	Song duration	? (Y for song as a whole)	Breeding experiment	“many”		Polygenic[b]	Vedenina et al. 2007
	Tibia strokes	?	Breeding experiment	1		Major loci[b]	Vedenina et al. 2007
C. brunneus/ jacobsi	Song syllables	Y	Breeding experiment	“few”		Major loci[b]	Saldamando et al. 2005
	Phrase length	Y	Breeding experiment	“few”		Major loci[b]	Saldamando et al. 2005
	Echeme length	Y	Breeding experiment	“many”		Polygenic[b]	Saldamando et al. 2005

Laupala paranigral kohalensis	Song pulse rate	Y	QTL	8	9.6	Polygenic[a,b]	Shaw et al. 2007
Homoptera							
Nilapavarta lugens: rice vs. *Leersia* feeding populations	Pulse repetition frequency	Y	Breeding experiment	4 or 5		Major loci[b]	Butlin 1996
Nilapavarta lugens: India vs. Australia rice feeding populations	pulse repetition frequency	Y	Breeding experiment	1.5 – 2		Major loci[b]	Butlin 1996

[1] "Y" denotes that authors claim trait is important in isolation. "N" denotes that authors claim trait is not important in isolation. "?" denotes that authors make no claim about the trait's impact on isolation.

[2] Maximum percentage of phenotypic variance explained by one locus.

[3] Authors' conclusion of genetic architecture. Superscripts denote the basis of authors' definitions of major loci versus polygenic architecture: [a]percent phenotypic variance explained by a single locus, [b]number of loci relative to total genome size.

Table 2-2: Genetic mechanisms controlling courtship differences within and between Drosophila species

Comparison	Species	Trait	Locus[1]	Locus overlap?	Comments	reference
Intraspecific	*D. melanogaster*	Interpulse interval (IPI)	2 (L) – 1 3 (L) – 2	N	Intraspecific differences through few loci	Gleason and Ritchie 2004
Interspecific	*D. simulans/ sechelia*		2 (R) – 4 3 (R) – 2		Interspecific differences through many loci	
Intraspecific	*D. melanogaster*	IPI	2 (L) – 1 3 (L) – 2	N	*D. pseudoobscura* and *persimilis* use different signals during mate choice.	Williams et al. 2001
Interspecific	*D. pseudoobscura/ persimilis*		X – 2 2 – 1			
Intraspecific	*D. montana*	Song traits	Autosomes	N		Hoikkala et al. 2005
Interspecific	*D. montana* subgroup		Autosome - ≥1 X – many			
Intraspecific	*D. virilis*	Pulse number (PN)	Autosomes Y	?	Possible overlap of autosomal loci yet to be done	Huttunen and Aspi 2003
Interspecific	*D. virilis/ lummei*		Autosomes			

Intraspecific	*D. virilis*	PN	X – 1 2 – 1 3 – 5 4 – 1	N	No autosomal loci involved in interspecific differences.	Huttunen et al. 2004
		Pulse train length (PTL)	3 – 4			
Interspecific	*D. virilis/ littoralis*	PN PTL	X (proximal end)			

[1] Format is as follows: chromosome (arm or region of chromosome) – number of loci in this region.

CHAPTER 3: COURTSHIP AND MATE DISCRIMINATION WITHIN AND BETWEEN SPECIES OF *TIMEMA* WALKING-STICKS

Devin Arbuthnott and Bernard J. Crespi. In Press, Animal Behaviour. Accepted Jan. 2009. Final Acceptance Feb. 2009.

Abstract

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.

Introduction

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 could be used in both intraspecific sexual selection and species recognition. Such a scenario could begin with courtship traits being used in intrapopulation mate choice. After a population split, sexual selection could push courtship traits and preferences in opposite directions in these populations, such that the two populations display different optima of courtship traits. Such divergence could continue through the process of speciation, to the point that newly formed species differ sufficiently in courtship traits that heterospecific individuals will not mate as a result of these differences. 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.

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. 3-1). 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 3-1.

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 3-1).

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. 3-2). 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. 3-2).

Interspecific mate discrimination

Table 3-2 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. 3-3). 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*.

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 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 *Timem*a 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; 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.

Acknowledgements

We thank Tanja Schwander and Patrik Nosil for help and advice in the field, and Todd Oakley for providing laboratory space. The FAB laboratory at Simon Fraser University contributed useful comments throughout the course of this project. Funding for this research was provided by a Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery grant to B.J.C., and an NSERC Canada Graduate Scholarship- Master’s to D.A.

References

Andersson, M. 1994. Sexual Selection. Princeton, NJ: Princeton University Press.

Arnqvist, G. & Rowe, L. 2005. Sexual Conflict. Princeton, NJ: Princeton University Press.

Bateman, P. W. & Fleming, P. A. 2006. Males are selective too: mating, but not courtship, with sequential females influences choosiness in male field crickets (Gryllus bimaculatus). Behavioral Ecology and Sociobiology, 59, 577-581.

Blows, M. W. & Allan, R. A. 1998. Levels of mate recognition within and between two Drosophila species and their hybrids. American Naturalist, 152, 826-837.

Boake, C. R. B. 2000. Flying apart: Mating behavior and speciation. Bioscience, 50, 501-508.

Boake, C. R. B. & Hoikkala, A. 1995. Courtship behavior and mating success of wild-caught Drosophila-silvestris males. Animal Behaviour, 49, 1303-1313.

Boake, C. R. B., DeAngelis, M. P. & Andreadis, D. K. 1997. Is sexual selection and species recognition a continuum? Mating behavior of the stalk-eyed fly Drosophila heteroneura. Proceedings of the National Academy of Sciences of the United States of America, 94, 12442-12445.

Boake, C. R. B., Andreadis, D. K. & Witzel, A. 2000. Behavioural isolation between two closely related Hawaiian Drosophila species: the role of courtship. Animal Behaviour, 60, 495-501.

Bonduriansky, R. 2001. The evolution of male mate choice in insects: a synthesis of ideas and evidence. Biology Reviews, 76, 305-339.

Brazner, J. C. & Etges, W. J. 1993. Pre-mating isolation is determined by larval rearing substrates in catophilic Drosophila mojavensis. II. Effects of larval substrates on time to copulation, mate choice and mating propensity. Evolutionary Ecology, 7, 605-624.

Butlin, R. K. & Ritchie, M. G. 1994. Behaviour and speciation. In: Behaviour and Evolution (Ed. by Slater, P. J. B. & T. R. Halliday), pp. 43-79. Cambridge, UK: Cambridge University Press.

Carracedo, M. C., Suarez, C. & Casares, P. 2000. Sexual isolation between Drosophila melanogaster, D-Simulans and D-mauritiana: sex and species specific discrimination. Genetica, 108, 155-162.

Chenoweth, S. F. & Blows, M. W. 2005. Contrasting mutual sexual selection on homologous signal traits in Drosophila serrata. American Naturalist, 165, 281-289.

Coyne, J. A. & Orr, H. A. 1989. Patterns of speciation in Drosophila. Evolution, 43, 362-381.

Coyne, J. A. & Orr, H. A. 1997. "Patterns of speciation in Drosophila" revisited. Evolution, 51, 295-303.

Coyne, J. A. & Orr, A. H. 2004. Speciation. Sunderland, MA: Sinauer Associates Publishers.

Engqvist, L. & Sauer, P. K. 2002. A life-history perspective on strategic mating effort in male scorpionflies. Behavioral Ecology and Sociobiology, 13, 632-636.

Etges, W. J., Over, K. F., De Oliveira, C. C. & Ritchie, M. G. 2006. Inheritance of courtship song variation among geographically isolated populations of Drosophila mojavensis. Animal Behaviour, 71, 1205-1214.

Gavrilets, S. & Boake, C. R. B. 1998. On the evolution of premating isolation after a founder event. American Naturalist, 152, 706-716.

Gowaty, P. A., Steinichen, R. & Anderson, W. W. 2003. Indiscriminate females and choosy males: Within- and between-species variation in Drosophila. Evolution, 57, 2037-2045.

Grant, B. R. & Grant, P. R. 2002. Lack of premating isolation at the base of a phylogenetic tree. American Naturalist, 160, 1-19.

Grant, P. R. & Grant, B. R. 1996. Speciation and hybridization in island birds. Philosophical Transactions of the Royal Society of London Series B-Biological Sciences, 351, 765-772.

Grant, P. R. & Grant, B. R. 1997. Genetics and the origin of bird species. Proceedings of the National Academy of Sciences of the United States of America, 94, 7768-7775.

Gray, D. A. 2005. Does courtship behavior contribute to species-level reproductive isolation in field crickets? Behavioral Ecology, 16, 201-206.

Hankison, S. J. & Morris, M. R. 2002. Sexual selection and species recognition in the pygmy swordtail, Xiphophorus pygmaeus: conflicting preferences. Behavioral Ecology and Sociobiology, 51, 140-145.

Henry, C. S., Wells, M. L. M. & Holsinger, K. E. 2002. The inheritance of mating songs in two cryptic, sibling lacewing species (Neuroptera : Chrysopidae : Chrysoperla). Genetica, 116, 269-289.

Higashi, M., Takimoto, G. & Yamamura, N. 1999. Sympatric speciation by sexual selection. Nature, 402, 523-526.

Hoikkala, A. & Welbergen, P. 1995. Signals and responses of females and males in successful and unsuccessful courtships of 3 Hawaiian lek-mating Drosophila species. Animal Behaviour, 50, 177-190.

Hoikkala, A., Crossley, S. & Castillo-Melendez, C. 2000. Copulatory courtship in Drosophila birchii and D. serrata, species recognition and sexual selection. Journal of Insect Behavior, 13, 361-373.

Kirkpatrick, M. 1982. Sexual selection and the evolution of female choice. Evolution, 36, 1-12.

Kvarnemo, C. & Ahnesjo, I. 1996. The dynamics of operational sex ratios and competition for mates. Trends in Ecology and Evolution, 11, 404-408.

Lande, R. 1981. Models of speciation by sexual selection on polygenic traits. Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences, 78, 3721-3725.

Law, J. H. & Crespi, B. J. 2002. The evolution of geographic parthenogenesis in Timema walking-sticks. Molecular Ecology, 11, 1471-1489.

Mayr, E. 1963. Animal Species and Evolution. Cambridge, MA: Belknap Press.

McPeek, M. A. & Gavrilets, S. 2006. The evolution of female mating preferences: Differentiation from species with promiscuous males can promote speciation. Evolution, 60, 1967-1980.

Nosil, P. 2004. Reproductive isolation caused by visual predation on migrants between divergent environments. Proceedings of the Royal Society of London Series B-Biological Sciences, 271, 1521-1528.

Nosil, P. 2007. Divergent host plant adaptation and reproductive isolation between ecotypes of Timema cristinae walking sticks. American Naturalist, 169, 151-162.

Nosil, P., Crespi, B. J. & Sandoval, C. P. 2002. Host-plant adaptation drives the parallel evolution of reproductive isolation. Nature, 417, 440-443.

Nosil, P., Crespi, B. J. & Sandoval, C. P. 2003. Reproductive isolation driven by the combined effects of ecological adaptation and reinforcement. Proceedings of the Royal Society of London Series B-Biological Sciences, 270, 1911-1918.

Nosil, P., Crespi, B. J., Gries, R. & Gries, G. 2007. Natural selection and divergence in mate preference during speciation. Genetica, 129, 309-327.

Peterson, M. A., Honchak, B. M., Locke, S. E., Beeman, T. E., Mendoza, J., Green, J., Buckingham, K. J., White, M. A. & Monsen, K. J. 2005. Relative abundance and the species-specific reinforcement of male mating preference in the Chrysochus (Coleoptera : Chrysomelidae) hybrid zone. Evolution, 59, 2639-2655.

Price, D. K. & Boake, C. R. B. 1995. Behavioral reproductive isolation in Drosophila-silvestris, D-heteroneura, and their F1 hybrids (Diptera, Drosophilidae). Journal of Insect Behavior, 8, 595-616.

Ritchie, M. G. & Gleason, J. M. 1995. Rapid evolution of courtship song pattern in Drosophila-willistoni sibling species. Journal of Evolutionary Biology, 8, 463-479.

Saarikettu, M., Liimatainen, J. O. & Hoikkala, A. 2005. Intraspecific variation in mating behaviour does not cause sexual isolation between Drosophila virilis strains. Animal Behaviour, 70, 417-426.

Sadowski, J. A., Grace, J. L. & Moore, A. J. 2002. Complex courtship Behavior in the striped ground cricket, Allonemobius socius (Orthoptera : Gryllidae): Does social environment affect male and female behavior? Journal of Insect Behavior, 15, 69-84.

Sandoval, C. P. 1994. DIifferential visual predation on morphs of Timema-cristinae (Phasmatodeae, Timemidae) and its consequences for host-range. Biological Journal of the Linnean Society, 52, 341-356.

Talyn, B. C. & Dowse, H. B. 2004. The role of courtship song in sexual selection and species recognition by female Drosophila melanogaster. Animal Behaviour, 68, 1165-1180.

Tanuja, M. T., Ramachandra, N. B. & Ranganath, H. A. 2001. Incipient sexual isolation in the nasuta-albomicans complex of Drosophila: mating preference in male-, female- and multiple-choice mating experiments. Journal of Biosciences, 26, 365-371.

Turner, G. F. & Burrows, M. T. 1995. A model of sympatric speciation by sexual selection. Proceedings of the Royal Society of London Series B-Biological Sciences, 260, 287-292.

Vishalakshi, C. & Singh, B. N. 2006. Sexual isolation between two sibling species of Drosophila: D. ananassae and D. pallidosa. Current Science, 90, 1003-1006.

Table 3-1: Courtship characteristics for *Timema*

Timema species	**Number of observations**	**Hosts**[1]	**Number of legs used in leg waving**	**Average Leg waving frequency (SD)**[2]	**% active courtship spent leg waving (SD)**[3]	**Average antenna waving frequency (SD)**[2]	**% 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)	

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

[2] Waving frequencies are measured as number of waves per second. Superscript letters denote 95% confidence interval groupings.

[3] % active courtship is calculated as time spent on behaviour/(time spent on leg waving + time spent on antenna waving).

Table 3-2: Propensity of mating behaviours for all male x female *Timema* species combinations in interspecific mate discrimination experiment

		Percentage of trials resulting in:		
Male	Female	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

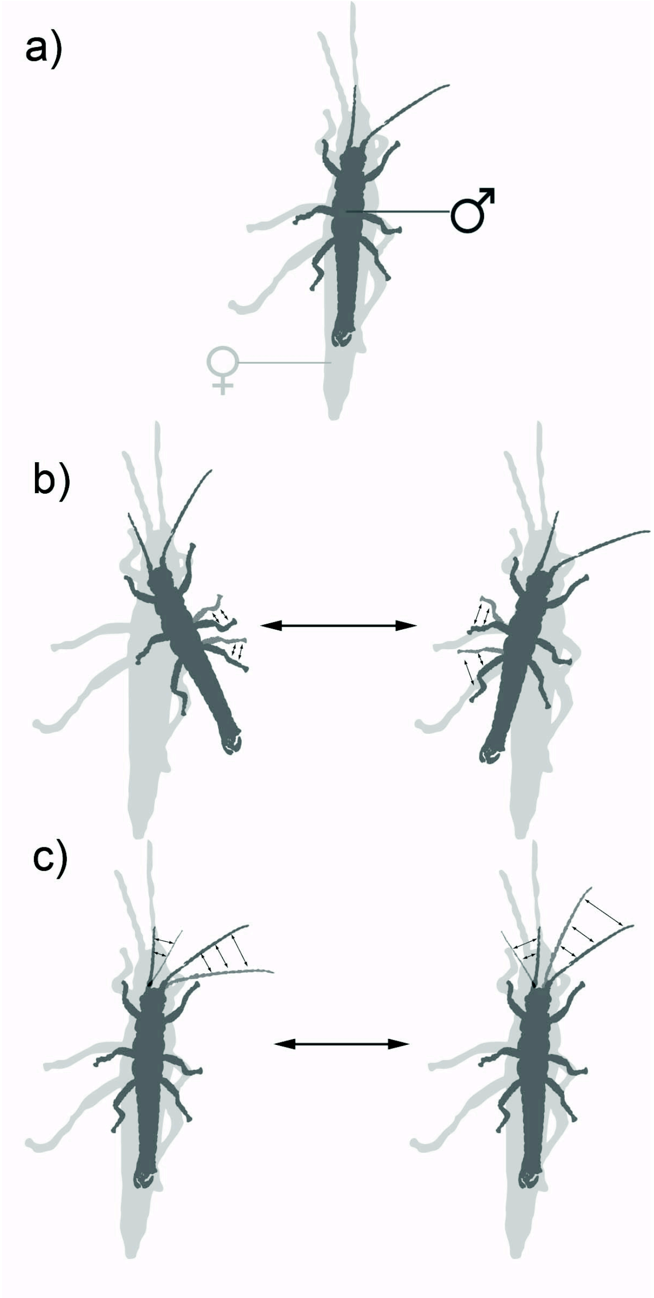


Figure 3-1: Dorsal view of typical *Timema* mating behaviour

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.

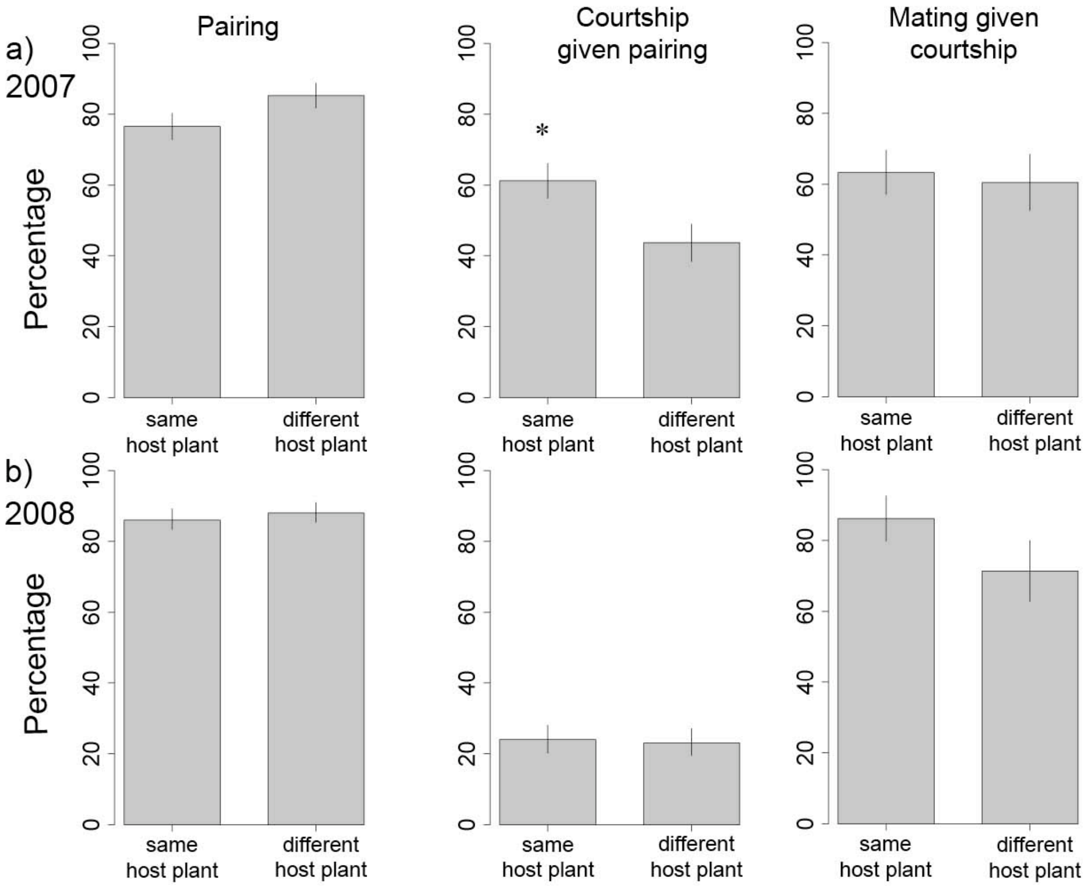


Figure 3-2: Percentage of *T. cristinae* no-choice trials that resulted in pairing, courtship given pairing, and mating given courtship

a) 2007 and b) 2008. Males courted different host-type females less than same host-type females in 2007 ($p = 0.025$), though there is no isolation between host-types in 2008.

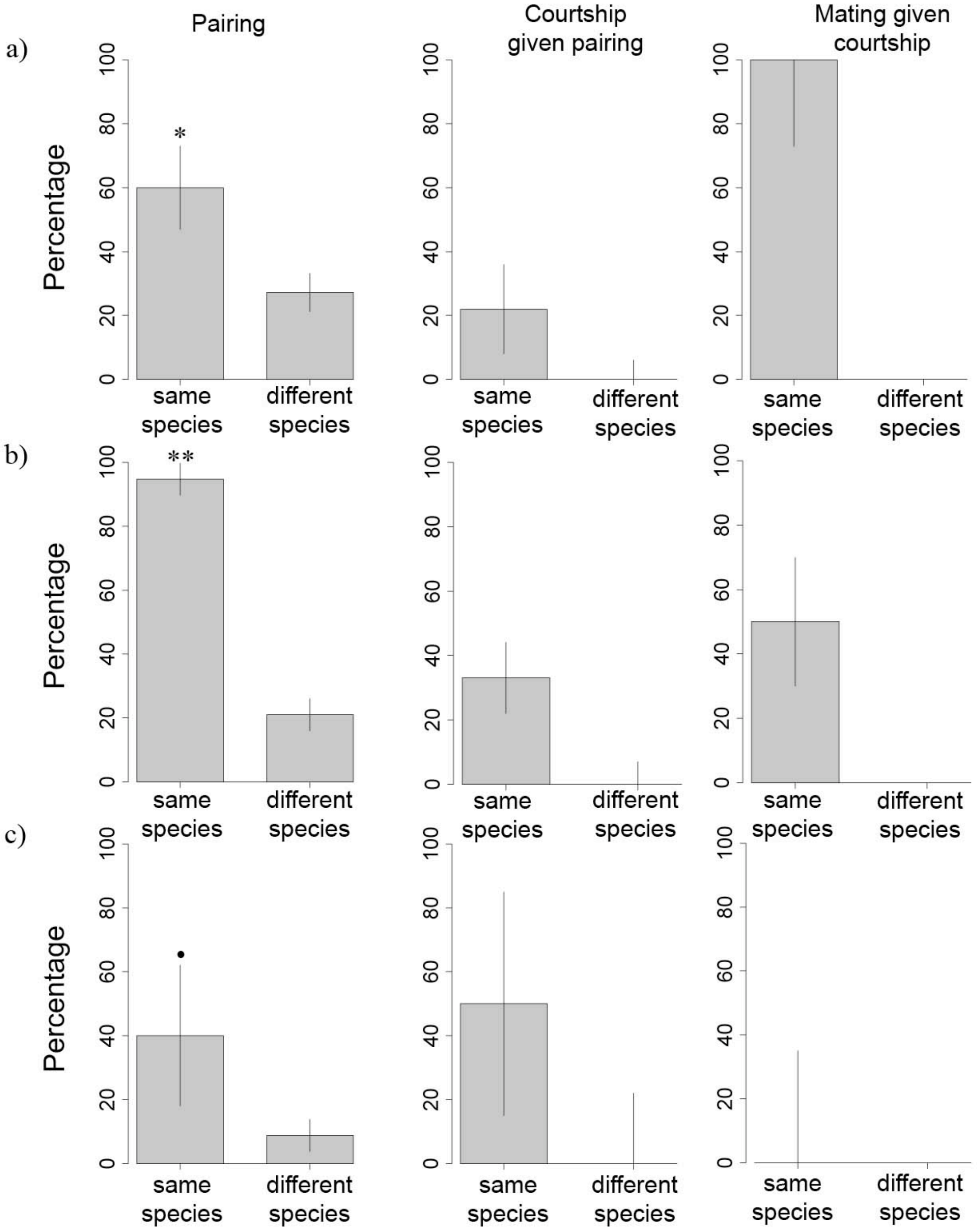


Figure 3-3: Percentage of no-choice trials that resulted in pairing, courtship given pairing, and mating given courtship for a) *T. cristinae*, b) *T. podura*, and c) *T. chumash*.

In all species, pairing is reduced when individuals are introduced to a heterospecific versus a conspecific.

CHAPTER 4: DIVERSIFICATION OF COURTSHIP AND GENITALIC CHARACTERS IN *TIMEMA* WALKING-STICKS

Abstract

We examined the patterns of phylogenetic diversification for courtship and external genitalic characters across 10 species of *Timema* walking-sticks, to infer the tempos and modes of character change in these sexual traits, and to draw inferences regarding the selective pressures underlying speciation and diversification in this clade. Rates of inferred change in each male courtship behaviour were proportional to speciation events rather than branch lengths, which implies that change in courtship occurs predominantly in association with the speciation process. By contrast, all male genitalic structures show a pattern of continuous change across evolutionary time, with divergence proportional to branch lengths. Functionally related characters show similar patterns of diversification, and are likely under similar selective pressures, but exhibit no evidence of coevolutionary change. Taken together, our findings suggest that diversification of courtship behaviour is mediated by processes that occur during speciation, such as divergent selection and mate choice in the context of population-level recognition, whereas diversification of genitalia occurs more or less continuously, most likely driven by forces of sexual selection or sexual conflict.

Introduction

Divergence in sexual traits often contributes to the evolution of reproductive isolation (Butlin and Ritchie 1994; Panhuis et al. 2001; Seddon 2005), and such divergence may be underlain by a number of mechanisms, including sexual selection, sexual conflict, species recognition, and ecological adaptation. The relative importance of these mechanisms can be inferred by analyzing character data from extant taxa in a phylogenetic framework, and interpreting these data in the context of information on microevolutionary change and character function (e.g. McPeek et al. 2008, 2009).

Sexual selection may underlie sexual trait diversification via processes whereby differing intraspecific mate preferences or traits affecting intrasexual competition confer a reproductive advantage on certain sexual characters, pushing character values in different directions for different species through time (see Andersson 1994 for review). By contrast, sexual conflict may promote trait diversification through sexually-antagonistic coevolution, whereby males and females develop adaptations and counteradaptations of sexual characters as both sexes seek to gain control over aspects of reproduction (see Arnqvist and Rowe 2005 for review). Sexual characters may also play a role in species recognition, which should select for species-specific characters exhibiting little intraspecific variation (e. g., McPeek et al. 2008). Finally, sexual traits may diversify in non-sexual, ecological contexts, if aspects of the environment influence the efficiency of communication or other interactions between males and females, or between competing males (Butlin and Ritchie 1994; Alexander et al. 1997). Features of the environment have been seen to shape courtship characters in a number of organisms, which show associations between courtship traits and components of the environment (Ryan and Wilczynski 1991; Sturmbauer et al. 1996; Johnson 2000; Zimmer et al. 2003; Henry and Wells 2004; Ferveur 2005; DaCosta et al. 2006; Shaw et al. 2007; Steinfartz et al. 2007). Determining the degree to which these diverse mechanisms underlie sexual trait diversification should lead to useful inferences regarding patterns and causes of speciation and evolutionary change.

Each of the four mechanisms described above for diversification in sexual traits generate predictions with respect to how sexual characters are expected to evolve, during both speciation and microevolutionary change. For example, both sexual selection and sexual conflict are expected to represent more or less continuous processes, such that interspecific or interpopulation phenotypic differences should tend to accumulate as a function of time rather than the number of speciation events (Andersson 1994; Polihronakis 2006). Species recognition, in contrast, should exert stabilizing selection for species-specific sexual characters between speciation events; therefore, the majority of phenotypic change should occur during speciation (McPeek et al. 2008). If sexual characters evolve in ecologically-adaptive contexts, then phenotypic change should be associated with aspects of the environment, and sexual phenotypes should be associated with components of the ecological niche (Butlin and Ritchie 1994; Alexander et al. 1997).

The mechanisms and selective forces underlying patterns of diversification for sexual traits can be evaluated by combining information on form and function of sexual characters with information on their phylogenetic patterns of diversification. For example, McPeek et al. (2008, 2009) used a combination of morphometric and phylogenetic data to infer that the majority of change in the external genitalia of *Enallagma* damselflies occurs during speciation events, a pattern concordant with the demonstrated species-recognition function of genitalia in this genus. Additional studies of the tempos and modes of diversification in sexual and species-recognition traits, across clades that differ in their mating systems, should lead to novel insights regarding the sexual and ecological factors that drive microevolutionary and macroevolutionary change in traits related to male-female interactions.

In this study, we analyzed patterns of phylogenetic diversification in male courtship behaviour, male external genitalic morphology, and male and female body morphology in *Timema* walking sticks, a genus of phytophagous insects that exhibits notable interspecific variation in male genital structures (Vickery 1993) and courtship (Arbuthnott and Crespi In Press). Our main goal is to evaluate alternative hypotheses for the tempos and modes of diversification in different forms of sexual and non-sexual traits in this genus, and to draw inferences regarding the selective pressures that have generated the observed patterns of variation within and between species.

Materials and Methods

Study system

The walking-stick genus *Timema* comprises about 20 species of phytophagous insects, 15 of which are sexual and 5 asexual, which are distributed primarily in California, but also in regions of Oregon, Arizona, Nevada, and northern Mexico (Vickery 1993, Law and Crespi 2002). The mating system of these species involves: (1) initial contact between a male and female, (2) pairing, whereby the male climbs onto the dorsal surface of the female, (3) male courtship behaviour, during which males rapidly vibrate (wave) their antennae, and their hind legs or middle and hinds legs, (4) copulation, which involves the male twisting the end of his abdomen beneath the female, using his tripartite, asymmetrical clasping external genitalia to engage, hold and manipulate the female during insertion of the aedaegus, and (5) long-term post-copulatory mate guarding, whereby the male rides on the female's dorsal surface, not in copula, for up to five days (Arbuthnott and Crespi in press). Females are capable of resisting males attempting to copulate by moving their abdomens away from male genitalia.

Sexual behaviour is integral to the evolution of reproductive isolation in *Timema*, given that premating isolation forms a major reproductive barrier between ecologically isolated populations within species (Nosil et al. 2002; Nosil 2004) and between species (Arbuthnott and Crespi in press). For example, *T. cristinae* exhibits a decreased incidence of courtship between individuals from populations on different host plants, whereas isolation between species (e.g. *T.*

cristinae, *T. chumash*, and *T. podura*) manifests as decreased pairing between interspecific males and females (Arbuthnott and Crespi in press). The functions of courtship variation among and within species have yet to be investigated, but species recognition and premating host-associated isolation in *Timema* appear to be mediated by chemical signals, given that mate discrimination occurs after antennal contact, but before pairing or courtship. Because isolation seems to occur before courtship is initiated, in both within- and between-species mate discrimination, observed interspecific differences in courtship (leg and antenna waving frequencies) apparently do not influence current reproductive isolation at either intermediate or complete levels of isolation in *Timema*. Also, *Timema* display multi-component sexual behaviours and complex, asymmetric external genitalia comprised of three structures used to clasp and manipulate females (asymmetric genitalia are a product of *Timema*'s asymmetric copulation position; Huber et al. 2007), which allows assessment of the degree to which functionally-linked traits share patterns of diversification.

Courtship observations

Timema were collected from March to June 2007 and 2008, and courtship was recorded in the laboratory. Recording procedures are given in Arbuthnott and Crespi (in press).

Phenotype-environment relationship

In *Timema*, we tested for an ecologically-adaptive role of courtship diversification by testing for associations between courtship phenotypes and host-plant structure. Differences in host-plant structure may constrain courting males' ability to move their legs, or may alter tactile signals transmitted during courtship. Changes in host-plant have a large impact on the evolution of reproductive isolation in *Timema* (Nosil et al. 2002, 2003; Nosil and Crespi 2006; Nosil 2007), and Arbuthnott and Crespi (in press) found that differences in courtship signals approach significance between individuals using separate host-plant species in *T. cristinae*. Host-plant species of *Timema* can be divided into two groups: those with relatively-broad leaves, and those with relatively needle-like leaves. We divide host plants into these two categories because elongated versus broad leaf shape influences cryptic morphology (Sandoval and Crespi 2008), which mediates reproductive isolation (Nosil et al. 2003, Nosil and Crespi 2004). To test for an adaptive role of courtship, we compared specific courtship characters between the broad leaf and needle leaf host-groups to determine if this aspect of ecology impacts courtship behaviour. To test for courtship changes associated with host plant shifts in a phylogenetically-controlled framework, we also performed independent contrasts analyses (Felsenstein 1985) to determine whether host leaf characteristics and courtship characteristics were evolutionarily correlated. To quantify host leaf structure, we obtained leaf length to width ratios from Jepson (1960) and Sargent (1933). Several *Timema* species can be found on more than one host plant (Vickery 1993), but for all species we used phenotypic data from the one or two most common host-type found in nature. We included two host-types in *T. cristinae* and *T. podura*, as these species are commonly found on both *Adenostoma fasciculatum* and

Ceanothus spinosus plants. To do this, we performed independent contrasts analyses four times, inputting all possible combinations of *Adenostoma* and *Ceanothus* averages for *T. cristinae* and *T. podura*. The host-type used for these two species did not influence our results, so we averaged the results over the four separate analyses.

Variation within species

To assess the level of intraspecific courtship variation, which can provide information on processes of intraspecific trait diversification, we quantified the behaviour of several individuals (4 – 27) in each species. We also collected samples from five populations of *T. cristinae* on different host plants (three *Adenostoma* and two *Ceanothus* populations); we tested for differences among these populations by performing an analysis of variance on leg and antenna waving frequencies, and used a Tukey's post-hoc test to determine the significance of each pairwise population difference. To assess the degree of intraspecific genitalic variation, we analyzed the genitalia of four separate *T. cristinae* populations.

Species-specificity of courtship

If courtship behaviour mediates species recognition, we would expect stabilizing selection for species-specific courtship phenotypes, which may tend to keep levels of within-species variation low for these traits. To evaluate the species-specificity of courtship, we performed a discriminant function analysis using four courtship characters: leg waving frequency, antenna waving frequency, per cent of active courtship spent on leg waving, and the number of legs used during leg waving. This analysis allows quantification of the degree to which species can be separated in multivariate space, using linear combinations of traits involved in courtship.

Genitalic morphology

Timema external genitalia comprise two asymmetric cerci (claspers) and an intradextral process. The two cerci are used for clasping the female's abdomen during copulation, while the intradextral process (on the sinistral side of the right cercus) aids in the opening of the female subgenital plate, which in turn allows the male to insert his aedaegus (internal genitalia). We generated and analyzed three-dimensional representations of genitalia using computer tomography (CT) for ten *Timema* species following the protocols described in McPeek et al. (2008). Two to eight males from each species were scanned, which produced digital slice images that were converted into three-dimensional representations using the program Amira (Mercury Computer Systems, Chelmsford, MA). Models were reconstructed as a triangular mesh with 20,000 data points, and analyzed using spherical harmonics (Shen and Makedon 2006). Spherical harmonics analysis is an extension of classical Fourier analysis, and reduces the complex, three-dimensional shape of the genitalic structures to a set of spherical harmonics coefficients. We used principal components analyses to reduce this high dimensional representation of the shapes to a small number of axes. All analyses were carried out for each of the three genitalic structures separately.

Morphological body characters

To compare the diversification patterns of courtship behaviour and genitalic morphology with the diversification patterns of morphological traits not involved in courtship or mating, we also analyzed a set of linear body traits, including length of the right hind and middle tibia, head width (eye-to-eye distance), thorax width (width of the widest portion of the thorax), and body length (anterior-most point of the head to the posterior-most point of the last abdominal segment). These measurements were carried out for males and females, and for two to 16 individuals per species, depending on the availability of samples.

Phylogenetics of diversification

Phylogenetic trees describing the relationships between the ten *Timema* species for which courtship, morphological, and genitalic data are available were constructed from mitochondrial COI sequences, 789 base pairs in length, using Mantophasmatodea COI (http://www.ncbi.nlm.nih.gov/nuccore/84488734) from Cameron et al. (2006) as an outgroup. The best nucleotide substitution model, general time reversible model with gamma distributed site rate variation and a proportion of invariable sites, was selected using hierarchical likelihood ratio tests (Nylander 2004). The model was fit to the sequence data using MrBayes version 3.1 (Ronquist and Huelsenbeck 2003). Markov chains were run for five million generations after attaining stationarity, as judged from the lack of a directional trend in likelihood over time. Chains were sampled every 1000 steps. The harmonic mean log likelihood of the stationary chain was calculated first with free branch lengths and again under the constraint of a global clock; a likelihood ratio test was unable to reject the hypothesis that the sequences have evolved under a clock-like process (Clock-like mean log likelihood = -3322.4, free mean log likelihood = -3316.9, $\chi 2 = 11.102$, d.f = 9, p = 0.26), such that branch lengths in this phylogeny can be interpreted as proportional to time.

The inferred phylogeny (Fig. 4-1) is fully compatible with previous studies of *Timema* (Law and Crespi 2002), in that *Timema* is split into a northern clade (*T. cristinae*, *T. landelsensis*, *T. knulli*, *T. poppensis*, *T. petita*, and *T. californicum*) and a southern clade (*T. chumash*, *T. bartmani*, *T. boharti*, and *T. podura*). Maximum parsimony and maximum likelihood analyses yielded the same topology for all well-supported nodes (nodes with Bayesian *a posteriori* values of 1, which exhibited ML and MP bootstrap values over 90%, for 200 replicates). Effects of uncertainty in tree topology on character change results were assessed by repeating analyses on the patterns of change of all courtship traits, and the PC1 scores of all genitalic structures using nine alternative trees, representing all permutations of branches exhibiting bootstrap or *a posteriori* support under 90%.

We used the program CoMET (Lee et al. 2006) which uses AIC criteria to determine which model of evolutionary change best characterizes the diversification of courtship, genitalia, and body-morphology traits. CoMET compares nine models of change, represented by a 3 x 3 table of possible model combinations, where the best model of the first group (which we refer to as the phylogenetic signal model) is paired with the best model of the second group (which we refer to as the tempo of change model). The first group of model classifications represents the phylogenetic pattern of phenotypic change, as pure-phylogenetic, non-phylogenetic, or punctuational. Under the pure-phylogenetic model, phenotypic change occurs along all branches, and phenotype therefore shows positive phylogenetic autocorrelation. The non-phylogenetic model disregards branching points, and essentially assumes a star phylogeny, such that phenotype shows no phylogenetic autocorrelation. The punctuational model assumes that at each bifurcation in the tree, one daughter branch retains the ancestral phenotype (zero change) and the other daughter branch changes.

The second group of model classifications represents the tempo of phenotypic change along branches as three possible models: distance, equal, and free. In the distance model, change is proportional to genetic distance (branch length). The equal model assumes that all branch lengths are equal, and phenotypic change is therefore independent of time since divergence. In the free model, branch lengths are any non-negative value calculated using maximum likelihood on trait values. The free model thus calculates phenotypic branch lengths, where lengths are proportional to the amount of phenotypic change, rather than as a function of genetic divergence. Under this model, phenotypic change is not proportional to time since divergence, and lineages diversify under different rates of phenotypic change.

We also tested for phylogenetic autocorrelation of each courtship, morphological, and genitalic trait using Phylogenetic Independence (Abouheif

1999), shuffling nodes of each topology 1000 times, and shuffling character data 1000 times. Phylogenetic independence outputs a correlation coefficient and the significance of this test statistic, and therefore gives a continuous measure of phylogenetic autocorrelation. CoMET, in contrast, estimates which discrete model of phylogenetic autocorrelation best represents the data.

To further assess the tempo of character diversification, we estimated Pagel's κ (Pagel 1997), which involves raising branch lengths to a range of exponents (κ), and determining what value best characterizes phenotypic change along a phylogenetic tree for each trait. If branches are best described by an exponent of zero, then all branch lengths are equal to one, and diversification is thus characterized by change only at speciation. If branches are best described by being raised to a power of one, then branch lengths are equal to genetic distances, and diversification is characterized as continuous change. We used a maximum likelihood search method to find the exponent that best characterizes phenotypic change along the *Timema* phylogeny, as described in McPeek et al. (2008). κ calculations determine what tempo of change best fits character divergence in a continuous way, by determining where in the continuum of speciational to continuous change a particular character lays, whereas CoMET assesses which end of this continuum best fits phenotypic data in a discrete way.

We performed independent contrasts analyses (Felsenstein 1985) to examine the evolutionary correlations of all courtship, genitalic, and other morphological traits, to evaluate hypotheses concerning the coevolution of functionally-related traits. We input species averages for leg waving frequency, percent of active courtship spent leg waving, antenna waving frequency, PC1 scores for left cercus, right cercus, and intradextral process shape, and female head width, thorax width, and body length. Characters with missing species averages were not used in these analyses. Bivariate correlations were calculated using the Contrast program of Phylip (Felsenstein 1985; Felsenstein 1989). We calculated the average correlation coefficients across 450 000 independent phylogenies, constraining well supported groupings, but allowing weakly supported groupings and branch lengths to vary. Testing across multiple trees may mask patterns found in only a minority of trees (Huelsenbeck and Rannala 2003), but this testing allows us to determine whether results are robust with respect to phylogenetic uncertainty.

Results

Phenotype-environment relationship

Species courtship phenotypes did not cluster based on structurally different host-groups (needle versus broad leaf plants; Fig. 4-2), and *Timema* species in the two host-plant groupings were not significantly different in leg waving frequency (Student's t-test; t = -0.45, df = 75, p = 0.66), antenna waving frequency (t = 0.77, df = 71, p = 0.44), or percent of active courtship spent on leg waving (t = -1.31, df = 69, p = 0.19). Similarly, results of independent contrasts analyses were non-significant for all pairs of traits: host leaf to width ratio and leg waving frequency (r = 0.49, p = 0.16), antenna waving frequency (r = -0.43, p = 0.2), or percent of active courtship spent on leg waving (r = -0.56, p = 0.1).

Adaptation to structurally different host plants thus does not seem to exert selective pressures on courtship behaviour.

Intraspecific variation in courtship

Many of the species examined exhibit a large range of courtship phenotypes, suggesting that intraspecific phenotypic variability is substantial (Fig. 4-2). *T. cristinae* showed significant interpopulation differences in antenna waving frequency (ANOVA; F = 3.82, df = 4, p = 0.018), but not in leg waving frequency (F = 0.53, df = 4, p = 0.72). In particular, there was a significant difference in antenna waving frequency between one *Adenostoma* population and one *Cenothus* population (p = 0.02), and the differences between two *Adenostoma* populations approach significance (p = 0.07). These findings suggest that among-population courtship differences within species of *Timema* may potentially mediate the evolution of courtship differences among species.

Species-specificity of courtship

There is noticeable overlap in our measured courtship characters among species (Fig. 4-2), such that these courtship traits cannot be described as strictly species-specific. Indeed, discriminant functions analysis assigned courtship characters correctly to species only 58.2 % of the time across the genus as a whole. Courtship phenotype was predictive of species identity in some cases, as suggested by a high percentage of correct species assignments in the discriminant functions analysis (*T. cristinae*: 85.2 %, *T. podura*: 77.8 %, *T. poppensis*: 83.3 %), but courtship phenotype displayed intermediate to low levels of species-specificity in the remainder of the species (*T. bartmani*: 50.0 %, *T. boharti*: 20 %, *T. californicum*: 20 %, *T. chumash*: 14.3 %, *T. knulli*: 60 %, *T. landelsensis*: 33.3 %, *T. petita*: 20 %). Low levels of species specificity can be a result of high levels of variance within species and overlap in phenotype between species. These data suggest that the courtship traits quantified here do not show high levels of species specificity, which is concordant with observations that reproductive isolation among species of *Timema* occurs prior to pairing and courtship (Arbuthnott and Crespi In Press).

Genitalic morphology

The first three PC axes for *Timema* left cercus account for 75% of phenotypic variance among species. PC1 represents the relative thickness and the curvature of the clasper, such that for low values the clasper resemble an oven mitt, and for high values the clasper becomes thinner and more forked. By contrast, PC2 represents the degree of twisting of the clasper (Fig. 4-3). For the right cercus, the first three PC axes account for 68% of phenotypic variance. As PC1 increases, the right clasper tip increases in forkedness, while PC2 represents relative thickness of the clasper and change in curvature at the tip (Fig. 4-3). For the intradextral process, the first three PC axes account for 59% of phenotypic variance. PC1 represents twisting of the process, while PC2 represents curvature (Fig. 4-3). Genitalic shape of all structures cluster well by species, though there is notable intraspecific variation (Figs 4-4 through 4-6).

PC1 of the left cercus separates the southern clade of *Timema* (*T. podura*, *T. chumash*, *T. bartmani*, and *T. boharti*) from the northern clade (all other

Timema species; Fig. 4-4). The northern clade clusters notably for right cercus morphology, while the southern clade is more varied (Fig.4- 5). The northern clade shows some clustering in intradextral process morphology, though the separation from southern clade species is not as extreme as the other two genitalic structures (Fig. 4-6).

Patterns of trait coevolution

The average and standard deviation of all courtship, morphological, and genitalic traits for each species are given in Table 4-1. By independent contrasts analyses, courtship characters are evolutionarily uncorrelated with one another ($p > 0.1$ for all pairwise correlations of courtship characters). Left cercus and right cercus shape, as well as left cercus and intradextral process shape do not show evolutionary correlation ($p > 0.1$), though right cercus and intradextral process shape do show a significant evolutionary correlation ($r = -0.83$, $p = 0.006$). Thorax width is evolutionarily correlated with both head width ($r = 0.69$, $p = 0.04$) and body length ($r = 0.80$, $p = 0.009$), and the correlation of body length with head width approaches significance ($r = 0.65$, $p = 0.06$). There is also a significant positive evolutionary correlation between host plant leaf length to width ratio and male body length ($r = 0.85$, $p = 0.004$).

Phylogenetics of trait diversification

For each courtship characteristic, CoMET analyses provided evidence for the same model of tempo, equal change (change not proportional to time since divergence), that fits each trait better than any other model (AIC difference > 3;

Table 4-2). Pagel’s κ values for antenna waving frequency and percent of active courtship spent leg waving are approximately 0, the κ value for leg waving frequency is 0.3, again suggesting courtship divergence is not proportional to branch lengths. By contrast, the model representing phylogenetic signal differed between traits: antenna waving frequency was best described by a model of pure-phylogenetic change (positive phylogenetic autocorrelation), percent of active courtship spent on leg waving was best described by a model of punctuational change, and leg waving frequency was best described by a model of non-phylogenetic change (no phylogenetic signal). From autocorrelation analysis using Phylogenetic Independence (Abouheif 1999), antenna waving frequency showed positive phylogenetic autocorrelation, while the percent of active courtship spent on leg waving shows significant negative autocorrelation (characters of more closely related species are more different than characters of distantly related species), and leg waving frequency shows a negative phylogenetic autocorrelation that approaches significance.

For all genitalic structures, CoMET analyses provided evidence for the same model of tempo, with change proportional to genetic distance, that fit each trait better than any other model (AIC difference > 2; Table 4-2). Similarly, a Pagel’s κ value of approximately 1 best fits phenotypic change in intradextral process shape, and a κ value of approximately 2 best fits change in left cercus shape. These values correspond to a model of genitalic character change as a more or less continuous process across macroevolutionary time. A κ value of 0.32 best fits change in right cercus shape, which suggests that there may be accelerated change during speciation events, but that phenotypic change also accumulates along branches in proportion to time. A phylogenetic-signal model of pure-phylogenetic change fits all of the genitalic traits, though a model of non-phylogenetic change also fits right cercus and intradextral process morphology. PC shape axes differ to some degree within the three genitalic structures, but these one or two models always best explain the majority of variance. From Phylogenetic Independence tests, the morphology of both cerci show significant positive phylogenetic autocorrelation,

For all non-genitalic morphological traits, CoMET provided one or two models that fit phenotypic changes better than other models (AIC difference > 3; Table 4-2). All morphological traits of both males and females were best fit by a tempo model of equal change. In agreement with the results from CoMET, κ values for all available non-genitalic (body) morphology are less than one. These results suggest a pattern of accelerated phenotypic change during speciation for courtship and body traits, though change may also accumulate through evolutionary time for some morphological body traits. The majority of traits fit a phylogenetic-signal model of non-phylogenetic change, though a model of pure-phylogenetic change was not significantly worse for hind tibia length of both sexes, female middle tibia length, male head width, or female thorax width, and a model of punctuated change also fits changes in male and female body length.

There was generally good agreement between our two metrics that quantify the tempo of character change (κ values and results of the CoMET model tests for tempo of change), and between our two metrics that measure phylogenetic autocorrelation (Phylogenetic Independence and the phylogenetic-signal test results from CoMET). Moreover, the same models of change were chosen for all characters across nine alternative phylogenies, indicating that the results are robust with respect to uncertainties in phylogeny.

Discussion

Courtship characters of male *Timema* walking-sticks demonstrate patterns of change primarily in proportion to the number of speciation events, whereas genitalic characters change more or less continuously. Courtship characters also show high levels of intraspecific variation and low species specificity, which is concordant with behavioural observations showing that courtship is not used for species recognition among well-differentiated species in extant populations (Arbuthnott and Crespi in press). However, our intraspecific analyses of of *T. cristinae* courtship provide evidence that among-population differences in courtship may be involved in the speciation process. In contrast to courtship traits, the pattern of change proportional to time inferred for genitalic characters implicates continuous selective forces, such as sexual selection or sexual conflict, in genitalic diversification. These findings indicate that courtship and genitalia traits undergo notably divergent patterns of macroevolutionary change, apparently in association with different microevolutionary forces during and between the processes of speciation.

Courtship diversification

Courtship behaviour effectively isolates members of separate populations and species in many taxa (e.g. Hoikkala and Welbergen 1995; Henry et al. 2002; Hoikkala et al. 2002), although in other taxa courtship does not play an obvious role in observed levels of reproductive isolation (e.g. Boake and Hoikkala 1995; Price and Boake 1995; Saarikettu et al. 2005). The effects of courtship behaviour on reproductive isolation among pairs or small sets of related species have been studied extensively, but the dynamics of phylogenetic change in courtship and other sexual behaviours have yet to be investigated in sufficient detail for robust comparative inferences to be drawn. In this study, we examined the patterns of evolutionary change in *Timema* courtship characters in order to test alternative hypotheses regarding the evolutionary forces acting on courtship, including hypotheses based on ecological adaptation, sexual selection/sexual conflict, and species recognition.

There is no evidence to support the hypothesis that diversification of courtship is a product of ecological adaptation or environment-specific communication optima, as there are no observable differences in courtship phenotype between host-plants differentiated by leaf shape. Previously, Arbuthnott and Crespi (in press) tested for differences in courtship characteristics between *T. cristinae* individuals found on *Adenostoma* and *Cenothus*, and found that antenna waving frequency differences approached significance ($p = 0.06$). In this study, we found significant differences between two specific populations of *T. cristinae* on different host plants, and differences between two populations on the same host plant approach significance, which suggests that the differences detected earlier indicate population-level rather than ecological differences. Although ecological shifts are an important factor in the evolution of reproductive isolation in *Timema* (Nosil et al. 2002, 2003; Nosil and Crespi 2006; Nosil 2007), and aspects of the environment influence the evolution of courtship traits in other animals (Ryan and Wilczynski 1991; Sturmbauer et al. 1996; Johnson 2000; Zimmer et al. 2003; Henry and Wells 2004; Ferveur 2005; DaCosta et al. 2006; Shaw et al. 2007; Steinfartz et al. 2007), ecology does not appear to exert direct selective pressures on the courtship behaviour of *Timema*.

Timema courtship traits show different patterns of phylogenetic autocorrelation, and no pairwise evolutionary correlations, but both CoMET and κ-based analyses indicate that phenotypic change tends to be independent of time for all courtship traits. Such discontinuous diversification suggests that change in courtship occurs primarily during speciation events, which in turn indicates that more or less continuous selective processes, such as sexual selection or sexual conflict, are unlikely to drive courtship diversification in *Timema* independently of speciation events. The courtship traits that we measured also show notable intraspecific variation, and discriminant function analysis indicates that these courtship traits are not highly species-specific. These findings suggest a lack of strong stabilizing selection on courtship traits between speciation events, which is concordant with previous observations that species-recognition occurs before males and females pair (Arbuthnott and Crespi in press), such that courtship does not mediate mate choice at the interpecific level.

A concentration of phenotypic change in short periods of evolutionary time might indicate that courtship is associated with early stages of speciation or population divergence, which would explain why there are significant differences in courtship between populations of *T. cristinae*. However, while courtship may function in the initiation of reproductive isolation, other processes such as reinforcement may be required to complete reproductive isolation, selecting for more efficient mate discrimination mechanisms. There are three lines of evidence supporting a hypothesis of reinforcement playing a role in *Timema* reproductive isolation; first, populations of *T. cristinae* are less likely to mate with conspecifics from different hosts if the two host-types are sympatric (Nosil et al. 2003). Second, males and females of the sympatric species *T. podura* and *T. chumash* never paired in no-choice mating trials, though both species would sometimes pair with allopatric *T. cristinae* individuals (Arbuthnott and Crespi in press). Third, the finding that some courtship traits (leg waving frequency and percent of active courtship spent leg waving) show negative phylogenetic autocorrelation is consistent with a pattern of notably-large phenotypic changes at speciation, driven by a reinforcement process. Further studies comparing the strength and mode of reproductive isolation in sympatric and allopatric populations or species are needed to support the hypothesis of reinforcement strengthening reproductive isolation in *Timema*.

Given that reinforcement may be important in *Timema* speciation, we postulate that mate discrimination should be selected to occur earlier as populations and species diverge, given benefits to both sexes from efficient avoidance of interspecific pairing and mating. In *Timema*, the mode of reproductive isolation has been inferred to change across evolutionary time scales, as isolation between ecologically divergent populations is mediated through decreased courtship, while species-level isolation occurs as decreased pairing (Arbuthnott and Crespi In Press).

Genitalic diversification

The evolutionary forces underlying the diversity of form and function in animal genitalia have been the subject of considerable debate for many years (see Alexander et al. 1997; Eberhard 1997 for example). Alternate hypotheses, such as lock-and-key, cryptic female choice, sperm competition, and sexual conflict have been described (Arnqvist 1997), but there is insufficient data regarding patterns of macroevolutionary change in genitalia to make inferences about the relative strength of these alternative mechanisms on a broad scale. McPeek et al. (2008) highlight the need for information on the dynamics of change, rather than quantifications of current diversity, in testing alternative hypotheses regarding genitalic diversification.

The majority of theory and empirical work on genitalia focuses on male intromittent organs, the structure that directly delivers sperm and seminal fluid. Such internal genitalia are commonly associated with complex, secondary sexual characters, such as structures for manipulating components of female reproductive structures. As these secondary sexual traits are only indirectly involved in the transfer of sperm, it is not always clear whether they should be considered genitalia. A useful framework for studying the genitalic evolution may be to consider genitalia as an integrated system of morphological structures which are used to transfer sperm and promote its use in females. Because *Timema*'s tripartite claspers are integral in the deployment of the aedeagus, and are closely associated with the aedeagus in a morphological context, we consider change in these claspers to be involved in the evolution of genitalia. However, the relationship between *Timema*'s internal and external genitalia have yet to be examined in detail.

In *Timema*, both CoMET and κ-based analyses indicate that change in external genitalic structures is best fit by a model of change proportional to evolutionary time, which suggests that change in *Timema* genitalic morphology is largely or entirely independent of speciation events. The κ value of the right cercus suggests that change may be accelerated to some degree during speciation events, but this calculation, as well as CoMET models, suggest that change is primarily continuous in this structure. Therefore, continuous selective pressures such as sexual selection, sexual conflict, or drift, may underlie genitalic shape diversification in *Timema*. Though there is no evolutionary correlation between the two cerci or the left cercus and the intradextral process, all genitalic structures show continuous change, which suggests that each structure evolves under similar evolutionary forces.

The function of *Timema* genitalia, aside from a general role in clasping the female's abdomen and prying the female's subgenital plate apart via the intradextral process, remain to be investigated. With respect to sexual selection, *Timema*'s external genitalia may stimulate females and encourage sperm use (copulatory courtship; Eberhard 1985, 2004). Genitalic shape may also change in the context of males more-effectively clasping onto intraspecific females, to facilitate efficient copulation or reduce copulation disruption from competing males. The inference that change in genitalic shape is best fit by a model of continuous divergergence agrees with a considerable body of work suggesting that genitalic diversification is mediated by sexual selection; for example, Arnqvist (1998) found that phylogenetic groups with strong sexual selection show up to twice as much genitalic diversity as monogamous groups, and Arnqvist (1997) concluded that sexual selection best fits pattern of genitalic diversity for most animals. Like waterstriders (which meet predictions of genitalic diversification through sexual conflict; Arnqvist and Thornhill 1998; Hosken and Stockley 2004), *Timema* genitalic morphology also shows intraspecific variation comparable to interspecific differences in some genitalic structures, which implicates continuous processes driving genitalic diversification.

Changes in *Timema* male genitalia may also represent sexually-antagonistic counteradaptations (Arnqvist and Rowe 1995, 2002; Ronn et al. 2007) to female morphology which inhibit males from grasping females or initiating copulation; such female morphology has not been observed in *Timema*, but females do sometimes reject male copulation attempts using side-to-side movements of their abdomen. Finally, if genitalic structures are capable of carrying out their specific functions regardless of shape differences, genitalic shape may represent a neutral character. By this hypothesis, changes in shape represent effects of drift, which might be expected to generate change proportional to time if speciation does not involve an acceleration of drift, for example via founder effects. Further data on copulatory behaviour, sperm transfer, and female sperm use are needed to discriminate between these and other hypotheses.

The tempo of genitalic evolution has been investigated in only one other study system, *Enallagma* damselflies, using the combined phylogenetic and morphometric approach used here. In these damselflies, males do not court females, male genitalic structures are highly species-specific, and behavioural observations provide strong evidence for a role of genitalic structures in species recognition (Robertson and Paterson 1982, Fincke 1982, Fincke et al. 2007, McPeek et al. 2008). In agreement with these observations, phylogenetic analyses demonstrated that male genitalic shape change of *Enallagma* is accelerated during speciation events (McPeek 2008), an inference further corroborated by correlated changes in associated morphology of female mating structures, also evolving in accordance with a model of speciational change (McPeek et al. 2009).

The diversification of genitalic morphology in *Enallagma* provides an interesting contrast to *Timema* (Table 4-3). In *Timema*, species-recognition takes place prior to copulation, which may remove genitalic structures from selective pressures underlying reproductive isolation. *Enallagma* damselflies lack courtship, and males of several species harass females and compete to mate (Fincke 1982, Fincke et al. 2007), which may select for a species-recognition function to both male and female genitalic morphology. Observed differences between these two systems suggest that species recognition systems trade off with systems underlying sexual selection and sexual conflict, which may subsequently limit the diversification patterns of separate traits. Because *Enallagma* do not show effective behavioural modes of species-recognition, genitalia serve this role in male-female interactions, and thus change primarily at speciation. *Timema*, in contrast, exhibit a mating system which allows for behavioural isolation early in male-female interactions, which may constrain behavioural mechanisms to change during speciation, but removes genitalia from selective pressures specific to reproductive isolation. Future work should investigate the relative roles of different sexual traits, and how potential functional trade-offs of these traits influence their patterns of diversification.

Phylogenetic signal of sexual traits

The degree of phylogenetic conservation in a character can provide useful information concerning convergent evolution and rates of character change across macroevolutionary time. Behavioural traits have been thought to evolve relatively quickly compared to morphological traits (Blomberg et al. 2003), but behavioural characters used in systematics do not show evidence of higher levels of homoplasy than morphological characters (de Queiroz and Wimberger

1993; Gittleman and Decker 1994), and mating behaviours are not more homoplasious than other behavioural or morphological traits (Foster et al. 1996).

A comprehensive review of relevant literature (Table 4-4) shows that closely related species are more similar in courtship than distantly related species in many groups, indicating that courtship traits commonly display positive phylogenetic autocorrelation. Despite this general pattern, some taxonomic groups show divergent patterns; for example, courtship generally displays positive phylogenetic autocorrelation for several groups of birds, while courtship in *Drosophila* does not, an observation consistent with rapid evolution of courtship in this genus (Etges et al. 2006). Such differences may result from a number of factors, such as the mode of character transmission (genetic versus learning mechanisms), the function of courtship (intraspecific mate choice versus species recognition), or morphological and physiological constraints affecting courtship behaviour.

The observed variation in phylogenetic autocorrelation among characters in *Timema* (Table 4-2) suggest that different characters show different rates and magnitudes of change in conjunction with speciation events and phyletic evolution. For example, all courtship characters change primarily during speciation events, though antenna waving frequency shows positive phylogenetic autocorrelation while other courtship characters show none. By contrast, all three genitalic structures show the same general patterns of change, in that they show positive phylogenetic autocorrelation (though autocorrelation is weak for the intradextral process) and diverge continuously. Each genitalic structure thus apparently accumulates change over time at a slow, phylogenetically conservative rate. In contrast to genitalia, body morphology characters show little evidence of phylogenetic autocorrelation, suggesting that body morphology changes during speciation events at magnitudes that mask phylogenetic relationships.

Correlated diversification of sexual phenotypes

Behaviour and morphology can either be subject to correlated evolution due to functional associations (Takami and Sota 2007), or they can be evolutionarily decoupled such that they evolve independently (e.g. Wiens 2000). Indeed, even apparently-associated traits can evolve under divergent trajectories, such as the genitalic components of male waterstriders, which function differently and are consequently subject to different selective pressures (Bertin and Fairbairn 2005). In *Timema,* evolutionary contrasts analyses indicate that the different traits involved in courtship, genitalic morphology, and body morphology appear to be evolving more or less independently of one another, despite the fact that, within each set of traits (courtship, genitalic, body morphology), all characters show the same broad patterns of evolutionary change.

As noted above, non-sexual morphological traits of *Timema*, such as leg length and thorax width, show a pattern of change primarily at speciation, similar to courtship characteristics (though κ indicates that these traits may also show some change between speciation events). This pattern of change may be indicative of the importance of body shape in adaptation to different host-plants via crypsis, which is a strong, well-documented selective force in this genus (Nosil and Crespi 2004). The evolutionary correlation between host plant leaf structure and male body length may be indicative of such a role for ecological adaptation in body shape, although experimental tests are required for robust hypothesis testing. It is not surprising that different types of traits show different patterns of change and reflect different selective pressures; however, our data indicate that functionally-similar characters can display different phylogenetic autocorrelations and show no evolutionary correlation while still showing the same tempos of evolutionary change. These results suggest that, while different characters are capable of evolving independently, functionally related traits are subject to similar large-scale selective pressures, and therefore show the same patterns of macroevolutionary change.

References

Abouheif, E. 1999. A method for testing the assumption of phylogenetic independence in comparative data. Evol. Ecol. Res. 1:895-909.

Alexander, R. D., D. C. Marshall, and J. R. Cooley. 1997. Evolutionary perspectives on insect mating. Pp. 4 - 31 *in* J. C. Choe, and B. J. Crespi, eds. The evolution of mating systems in insects and arachnids. Cambridge University Press, Cambridge, UK.

Alonso-Pimentel, H., G. Spangler, and W. B. Heed. 1995. Courtship sounds and behaviour of the two saguaro-breeding Drosophila and their relatives. Anim. Behav. 50:1031 - 1039.

Andersson, M. 1994. Sexual Selection. Princeton University Press, Princeton, NJ.

Arbuthnott, D., and B. J. Crespi. In Press. Courtship and mate discrimination within and between species of Timema walking-sticks. Anim. Behav.

Arnqvist, G. 1997. The evolution of animal genitalia: Distinguishing between hypotheses by single species studies. Biol. J. Linnean Soc. 60:365-379.

Arnqvist, G. 1998. Comparative evidence for the evolution of genitalia by sexual selection. Nature 393:784-786.

Arnqvist, G., and L. Rowe. 1995. Sexual conflict and arms races between the sexes - a morphological adaptaion for control of mating in a female insect. Proc. R. Soc. Lond. Ser. B-Biol. Sci. 261:123-127.

Arnqvist, G., and L. Rowe. 2002. Correlated evolution of male and female morphologies in water striders. Evolution 56:936-947.

Arnqvist, G., and L Rowe. 2005. Sexual Conflict. Princeton University Press, Princeton, NJ.

Arnqvist, G., and R. Thornhill. 1998. Evolution of animal genitalia: patterns of phenotypic and genotypic variation and condition dependence of genital and non-genital morphology in water strider (Heteroptera : Gerridae : Insecta). Genet. Res. 71:193-212.

Bertin, A., and D. J. Fairbairn. 2005. One tool, many uses: precopulatory sexual selection on genital morphology in Aquarius remigis. J. Evol. Biol. 18:949-961.

Blomberg, S. P., T. Garland, and A. R. Ives. 2003. Testing for phylogenetic signal in comparative data: Behavioural traits are more labile. Evolution 57:717-745.

Boake, C. R. B., and A. Hoikkala. 1995. Courtship behaviour and mating success of wild-caught Drosophila-silvestris males. Anim. Behav. 49:1303-1313.

Boake, C. R. B., M. P. DeAngelis, and D. K. Andreadis. 1997. Is sexual selection and species recognition a continuum? Mating behaviour of the stalk-eyed fly Drosophila heteroneura. Proc. Natl. Acad. Sci. U. S. A. 94:12442-12445.

Butlin, R. K., and M. G. Ritchie. 1994. Behaviour and speciation. Pp. 43-79 *in* P. J. B. a. H. Slater, T. R., ed. Behaviour and Evolution. Cambridge University Press, Cambridge, UK.

Cameron, S. L., Barker, S. C., and Whiting, M. F. 2006. Mitochondrial genomics and the new insect order Mantophasmatodea. Mol. Phylogenet. Evol. 38: 274-279.

Carracedo, M. C., C. Suarez, and P. Casares. 2000. Sexual isolation between Drosophila melanogaster, D-Simulans and D-mauritiana: sex and species specific discrimination. Genetica 108:155-162.

DaCosta, M. A., P. Larson, J. P. Donahue, and S. J. Weller. 2006. Phylogeny of milkweed tussocks (Aretiidae : Arctiinae : Phaegopterini) and its implications for evolution of ultrasound communication. Ann. Entomol. Soc. Am. 99:723-742.

de Queiroz, A., and P. H. Wimberger. 1993. The usefulness of behaviour for phylogeny estimation - levels of homoplasy in behavioural and morphological characters. Evolution 47:46-60.

Eberhard, W. G. 1985. Sexual selection and animal genitalia. Harvard University Press, Cambridge, MA.

Eberhard, W. G. 1997. Sexual selection by cryptic female choice in insects and arachnids. Pp. 32-57 *in* J. C. Choe, and B. J. Crespi, eds. The evolution of mating systems in insects and arachnids. Cambridge University Press, Cambridge, UK.

Eberhard, W. G. 2004. Rapid divergent evolution of sexual morphology: Comparative tests of antagonistic coevolution and traditional female choice. Evolution 58:1947-1970.

Felsenstein, J. 1985. Phylogenies and the comparative method. Am. Nat. 125(1): 1-15.

Felsenstein, J. 1989. PHYLIP - Phylogeny Inference Package (Version 3.2). Cladistics 5: 164-166.

Ferveur, J. F. 2005. Cuticular hydrocarbons: Their evolution and roles in Drosophila pheromonal communication. Behav. Genet. 35:279-295.

Fincke, O. M. 1982. Lifetime mating success in a natural population of the damselfly *Enellagma hageri* (Walsh) (Odanata, Coenagrionidae). Behav. Ecol. Soc. 10:293-302.

Fincke, O. M., A. Farevieille, and T. D. Schulz. 2007. Lack of innate preference for morph and species identity in mate-searching Enallagma damselflies. Behav. Ecol. Soc. 61:1121-1131.

Foster, S. A., W. A. Cresko, K. P. Johnson, M. U. Tlusty, and H. E. Willmott. 1996. Patterns of homoplasy in behavioural evolution. Pp. 245-269 *in* M. J. Sanderson, and L. Hufford, eds. Homoplasy: the recurrence of similarity in evolution. Academic Press, San Diego, CA.

Gittleman, J. L., and D. M. Decker. 1994. The phylogeny of behaviour. Pp. 80-105 *in* P. J. B. a. H. Slater, T. R., ed. Behaviour and Evolution. Cambridge University Press, Cambridge, UK.

Gleason, J. M., and M. G. Ritchie. 1998. Evolution of courtship song and reproductive isolation in the Drosophila willistoni species complex: Do sexual signals diverge the most quickly? Evolution 52:1493-1500.

Hankison, S. J., and M. R. Morris. 2002. Sexual selection and species recognition in the pygmy swordtail, Xiphophorus pygmaeus: conflicting preferences. Behav. Ecol. Sociobiol. 51:140-145.

Harrison, R. G., and S. M. Bogdanowicz. 1995. Mitochondrial-DNA phylogeny of North-American field crickets - perspectives on teh evolution of life-cycles, songs, and habitat associations. J. Evol. Biol. 8:209-232.

Henry, C. S., and M. L. M. Wells. 2004. Adaptation or random change? The evolutionary response of songs to substrate properties in lacewings (Neuroptera : Chrysopidae : Chrysoperla). Anim. Behav. 68:879-895.

Henry, C. S., M. L. M. Wells, and K. E. Holsinger. 2002. The inheritance of mating songs in two cryptic, sibling lacewing species (Neuroptera : Chrysopidae : Chrysoperla). Genetica 116:269-289.

Hoikkala, A., K. Y. Kaneshiro, and R. R. Hoy. 1994. Courtship songs of the picture-winged Drosophila-plantibia subgroup species. Anim. Behav. 47:1363-1374.

Hoikkala, A., K. Klappert, and D. Mazzi. 2005. Factors affecting male song evolution in Drosophila montana. Pp. 225-250. Current Topics in Developmental Biology, Vol 67. Elsevier Academic Press Inc, San Diego.

Hoikkala, A., M. Saarikettu, S. Paallysaho, and M. G. Ritchie. 2002. Co-evolution of male courtship songs and female song preferences in Drosophila virilis group species. Behav. Genet. 32:470-470.

Hoikkala, A., and P. Welbergen. 1995. Signals and responses of females and males in successful and unsuccessful courtships of 3 Hawaiian lek-mating Drosophila species. Anim. Behav. 50:177-190.

Hosken, D. J., and P. Stockley. 2004. Sexual selection and genital evolution. Trends Ecol. Evol. 19:87-93.

Huber, B. A., Sinclair, B. J., and Scmitt, M. 2007. The evolution of asymmetric genitalia in spiders and insects. Biol. Rev. 82: 647-698.

Huelsenbeck, J.P. and Rannala, B. 2003. Detecting Correlation between Characters in a Comparative Analysis with Uncertain Phylogeny. Evolution 57(6): 1237-1247.

In Den Bosch, H. A. J., and M. Zandee. 2001. Courtship behaviour in lacertid lizards: Phylogenetic interpretations of the Lacerta kulzeri complex (Reptilia : Lacertidae). Neth. J. Zool. 51:263-284.

Jepson, W. L. 1960. A manual of the flowering plants of California. University of California Press, Berkeley CA.

Johnson, K. P. 2000. The evolution of courtship display repertoire size in the dabbling ducks (Anatini). J. Evol. Biol. 13:634-644.

Kitaura, J., and K. Wada. 2006. Evolution of waving display in brachyuran crabs of the genus Ilyoplax. J. Crustac. Biol. 26:455-462.

Law, J. H. & Crespi, B. J. 2002. The evolution of geographic parthenogenesis in Timema walking-sticks. Mol. Ecol., 11, 1471-1489.

Lee, C., S. Blay, A. O. Mooers, A. Singh, and T. H. Oakley. 2006. CoMET: a mesquite package for comparing models of continuous character evolution on phylogenies. Evolutionary Bioinformatics Online 2:193 - 196.

Maddison, W., and M. Hedin. 2003. Phylogeny of Habronattus jumping spiders (Araneae : Salticidae), with consideration of genital and courtship evolution. Syst. Entomol. 28:1-21.

Markow, T. A., and P. M. O'Grady. 2005. Evolutionary genetics of reproductive behaviour in Drosophila: Connecting the dots. Annu. Rev. Genet. 39:263-291.

McPeek, M. A., L. Shen, and H. Farid. 2009. The correlated evolution of three-dimensional reproductive structures between male and female damselflies. Evolution 63:73-83.

McPeek, M. A., L. Shen, J. Z. Torrey, and H. Farid. 2008. The tempo and mode of three-dimensional morphological evolution in male reproductive structures. Am. Nat. 171:E158-E178.

Mead, L. S., and P. A. Verrell. 2002. Evolution of courtship behaviour patterns and reproductive isolation in the Desmognathus ochrophaeus complex. Ethology 108:403-427.

Nosil, P. 2004. Reproductive isolation caused by visual predation on migrants between divergent environments. Proc. R. Soc. Lond. Ser. B-Biol. Sci. 271:1521-1528.

Nosil, P. 2007. Divergent host plant adaptation and reproductive isolation between ecotypes of Timema cristinae walking sticks. Am. Nat. 169:151-162.

Nosil, P., and B. J. Crespi. 2004. Does gene flow constrain adaptive divergence or vice versa? A test using ecomorphology and sexual isolation in Timema cristinae walking-sticks. Evolution 58:102-112.

Nosil, P., and B. J. Crespi. 2006. Ecological divergence promotes the evolution of cryptic reproductive isolation. Proc. R. Soc. B-Biol. Sci. 273:991-997.

Nosil, P., B. J. Crespi, and C. P. Sandoval. 2002. Host-plant adaptation drives the parallel evolution of reproductive isolation. Nature 417:440-443.

Nosil, P., B. J. Crespi, and C. P. Sandoval. 2003. Reproductive isolation driven by the combined effects of ecological adaptation and reinforcement. Proc. R. Soc. Lond. Ser. B-Biol. Sci. 270:1911-1918.

Nyalander, J. A. A. 2004. MrModeltest v2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University.

Pagel, M. 1997. Inferring evolutionary processes from phylogenies. Zoologica Scripta 26: 331-348.

Panhuis, T. M., R. Butlin, M. Zuk, and T. Tregenza. 2001. Sexual selection and speciation. Trends Ecol. Evol. 16:364-371.

Polihronakis, M. 2006. Morphometric analysis of intraspecific shape variation in male and female genitalia of Phyllophaga hirticula (Coleoptera : Scarabaeidae : Melolonthinae). Ann. Entomol. Soc. Am. 99:144-150.

Price, D. K., and C. R. B. Boake. 1995. Behavioural reproductive isolation in Drosophila-silvestris, D-heteroneura, and their F1 hybrids (Diptera, Drosophilidae). J. Insect Behav. 8:595-616.

Price, J. J., and S. M. Lanyon. 2002. Reconstructing the evolution of complex bird song in the oropendolas. Evolution 56:1514-1529.

Prum, R. O. 1990. Phylogenetic analysis of the evolution of display behaviour in the neotropical manakins (Aves, Pipridae). Ethology 84:202-231.

Prum, R. O. 1998. Sexual selection and the evolution of mechanical sound production in manakins (Aves : Pipridae). Anim. Behav. 55:977-994.

Ritchie, M. G., and J. M. Gleason. 1995. Rapid evolution of courtship song pattern in Drosophila-willistoni sibling species. J. Evol. Biol. 8:463-479.

Robertson, H. M. and H. E. H. Paterson. 1982. Mate recognition and mechanical isolation in *Enallagma* damselflies (Odanata: Coenagrionidae). Evolution 36: 243-250.

Ronn, J., M. Katvala, and G. Arnqvist. 2007. Coevolution between harmful male genitalia and female resistance in seed beetles. Proc. Natl. Acad. Sci. U. S. A. 104:10921-10925.

Ronquist, F. and J. P. Huelsenbeck. 2003. MRBAYES 3: Bayesion phylogenetic inference under mixed models. Bioinformatics 19: 1572-1574.

Ryan, M. J., and A. S. Rand. 1995. Female responses to ancestral advertisement calls in Tungara frogs. Science 269:390-392.

Ryan, M. J., A. S. Rand, and L. A. Weigt. 1996. Allozyme and advertisement call variation in the tungara frog, Physalaemus pustulosus. Evolution 50:2435-2453.

Ryan, M. J., and W. Wilczynski. 1991. Evolution of intraspecific variation in the advertisement call of a cricket frog (Acris-crepitans, Hylidae). Biol. J. Linnean Soc. 44:249-271.

Saarikettu, M., J. O. Liimatainen, and A. Hoikkala. 2005. Intraspecific variation in mating behaviour does not cause sexual isolation between Drosophila virilis strains. Anim. Behav. 70:417-426.

Sandoval, C. P. and Crespi, B. J. 2008. Adaptive evolution of cryptic coloration: the shape of host plants and dorsal stripes in Timema walking-sticks. *Biological Journal of the Linnean Society* **94**: 1-5.

Sargent, C. S. 1933. Manual of the trees of North America. The Riverside Press, New York, NY.

Seddon, N. 2005. Ecological adaptation and species recognition drives vocal evolution in neotropical suboscine birds. Evolution 59:200-215.

Shaw, K. A., M. L. Scotti, and S. A. Foster. 2007. Ancestral plasticity and the evolutionary diversification of courtship behaviour in threespine sticklebacks. Anim. Behav. 73:415-422.

Shen, L., and F. Makedon. 2006. Spherical mapping for processing of 3D closed surfaces. Image Vis. Comput. 24:743-761.

Slikas, B. 1998. Recognizing and testing homology of courtship displays in storks (Aves : Ciconiiformes : Ciconiidae). Evolution 52:884-893.

Steinfartz, S., S. Vicario, J. W. Arntzen, and A. Caccone. 2007. A Bayesian approach on molecules and behaviour: Reconsidering phylogenetic and evolutionary patterns of the Salamandridae with emphasis on Triturus newts. J. Exp. Zool. Part B 308B:139-162.

Sturmbauer, C., J. S. Levinton, and J. Christy. 1996. Molecular phylogeny analysis of fiddler crabs: Test of the hypothesis of increasing behavioural complexity in evolution. Proc. Natl. Acad. Sci. U. S. A. 93:10855-10857.

Takami, Y., and T. Sota. 2007. Rapid diversification of male genitalia and mating strategies in Ohomopterus ground beetles. J. Evol. Biol. 20:1385-1395.

Vickery, V. R. 1993. Revision of Timema Scudder (Phasmatoptera, Timematodea) including 3 new species. Can. Entomol. 125:657-692.

Wiens, J. J. 2000. Decoupled evolution of display morphology and display behaviour in phrynosomatid lizards. Biol. J. Linnean Soc. 70:597-612.

Zimmer, M., O. Diestelhorst, and K. Lunau. 2003. Courtship in long-legged flies (Diptera: Dolichopodidae): function and evolution of signals. Behav. Ecol. 14:526-530.

Table 4-1: Average courtship and morphological values for ten Timema species. Standard deviations are given in brackets.

Species	**bartmani**	**boharti**	**californicum**	**chumash**	**cristinae**	**knulli**	**landelsensis**	**petita**	**podura**	**poppensis**
Hosts[1]	F (needle)	E, F (needle)	G (broad)	D, E (both)	D, E (both)	B (needle)	C (broad)	D (broad)	D, E (both)	A (needle)
Legs used during leg waving	2 pairs	2 pairs	2 pairs	1 pair	1 pair	2 pairs	2 pairs	2 pairs	1 pair	2 pairs
Average leg waving frequency[2]	3. 858 (1.054)	5.292 (2.85)	4.15 (0.92)	3.861 (1.19)	3.412 (1.2)	4.578 (0.63)	3.67 (1.24)	4.447 (0.72)	1.333 (1.06)	2.476 (1.0)
Per cent active courtship spent leg waving[3]	80 (14)	27 (14)	39.7 (19)	28.2 (18)	29.6 (18)	46.6 (16)	24.2 (12)	20.2 (10)	7.4 (15)	20.6 (10)
Average antenna waving frequency[2]	2.16 (0.38)	1.693 (0.66)	1.629 (0.24)	2.032 (0.3)	1.465 (0.41)	1.048 (0.19)	1.566 (0.23)	1.554 (0.47)	2.168 (0.44)	1.08 (0.2)
Female hind tibia length	3.52 (0.38)		3.47 (0.08)	3.77 (0.2)	2.85 (0.5)	4.91 (0.06)	3.65 (0.13)	3.19	2.91 (0.13)	4.25 (0.15)
Male hind tibia length			3.11 (0.06)	3.01 (0.1)	2.55 (0.14)	4.0 (0.19)	3.22 (0.25)	2.52	2.5 (0.12)	3.58 (0.09)

Female middle tibia length	2.44 (0.17)	2.31 (0.19)	2.44 (0.04)	2.65 (0.06)	2.34 (0.11)	3.3 (0.12)	2.64 (0.25)	2.15	2.21 (0.18)	2.95 (0.19)
Male middle tibia length		2.08	2.37 (0.08)	2.27 (0.18)	1.96 (0.15)	2.85 (0.22)	2.3 (0.13)	1.79	1.98 (0.08)	2.46 (0.05)
Female head width	2.04 (0.22)	1.95 (0.09)	2.04 (0.05)	2.29 (0.12)	1.79 (0.12)	2.31 (0.03)	2.1 (0.09)	1.75	1.92 (0.08)	2.12 (0.1)
Male head width		1.79 (0.24)	1.86 (0.05)	2.01 (0.06)	1.56 (0.11)	1.94 (0.03)	1.77 (0.07)	1.4	1.7 (0.08)	1.88 (0.11)
Female thorax width	3.41 (0.32)	4.13 (0.34)	3.79 (0.1)	4.49 (0.29)	3.9 (0.37)	4.41 (0.27)	4.15 (0.31)	3.58	3.82 (0.18)	3.95 (0.36)
Male thorax width		2.86 (0.32)	2.77 (0.02)	2.86 (0.08)	2.69 (0.15)	2.94 (0.56)	2.76 (0.16)	2.4	2.75 (0.1)	2.76 (0.01)
Female body length	13.93 (1.31)	20.11 (0.13)	18.51 (1.79)	14.72 (1.62)	18.85 (1.69)	20.01 (2.02)	20.17 (0.63)	12.78	16.42 (2.27)	19.95 (2.23)
Male body length		16.23 (1.43)	13.05 (1.07)	9.79 (0.82)	12.09 (1.56)	16.09 (2.24)	14.69 (0.57)	11.14	11.73 (0.97)	16.29 (1.48)
Left cercus PC1 score	-0.0076	-0.0086	0.005	-0.0097	0.0053	0.0044	0.0059	0.0038	-0.0094	0.003
Left cercus PC2 score	0.0012	-0.0002	-0.004	0.00067	0.0034	-0.0029	-0.0027	-0.0027	-0.00062	-0.0039

Left cercus PC3 score	0.0015	0.0013	-0.00057	-0.0021	-0.00022	-0.0015	-0.003	0.004	0.0016	0.0018
Right cercus PC1 score	-0.0042	-0.002	0.0032	0.002	0.0015	0.001	0.00076	0.002	-0.0069	0.0029
Right cercus PC2 score	-0.0027	0.00085	0.0011	-0.0015	0.00005	0.0021	0.0012	0.00099	-0.004	0.0017
Right cercus PC3 score	0.0003	-0.0003	0.00099	-0.0039	0.0013	0.00003	0.00087	0.0004	0.0016	0.00009
Intradextral process PC1 score	0.0042	0.0033	0.00003	-0.0015	-0.0028	0.00052	0.0012	0.00086	0.0072	-0.0019
Intradextral process PC2 score	0.0003	-0.0006	0.0023	0.0062	-0.002	-0.0021	0.00015	-0.00053	-0.00097	-0.0022
Intradextral process PC3 score	0.00031	0.0028	0.00004	-0.00036	0.0013	-0.004	0.00057	0.00066	-0.00078	-0.0036

[1] Hosts are noted as in Law and Crespi (2002): A- *Pseudotsuga menziesii*, B- *Sequoia sempervirens*, C- *Arctostaphylos* species (manzanita), D- *Ceanothus* species, E- *Adenostoma fasiculatum*, F- *Abies concolor*, G- *Quercus* species. Species' host plants are divided into needle or broad leaf plants. A, B, E, and F are needle leaf plants, while C, D, and G are broad leaf plants.

[2] Waving frequencies are measured as number of waves per second. Superscript letters denote 95% confidence interval groupings.

[3] % active courtship is calculated as time spent on behaviour/(time spent on leg waving + time spent on antenna waving).

Table 4-2: Phylogenetic patterns of change for all Timema characters. All courtship and most body morphology meet predictions of speciational change, while all genitalic structures meet predictions of continuous change.

Trait	**Phylogenetic auto-correlation**[1]	**Phylogenetic auto-correlation p-value**	**CoMET model phylogenetic signal**[2]	**CoMET model tempo of trait change**[2]	**κ** [3]
Courtship traits					
Leg waving frequency	-0.2226	0.069	Non-phylogenetic	Equal	0.378
Per cent active courtship spent leg waving	-0.3102	0.035	Punctuated	Equal	0
Antenna waving frequency	0.5444	0.011	Pure-phylogenetic	Equal	0
Body morphology					
Female hind tibia length	0.025	0.317	Non-phylogenetic, Pure-phylogenetic	Equal	
Male hind tibia length	-0.139	0.48	Non-phylogenetic, Pure-phylogenetic	Equal	
Female middle tibia length	-0.0459	0.468	Non-phylogenetic, Pure-phylogenetic	Equal	0
Male middle tibia length	-0.1112	0.321	Non-phylogenetic	Equal	
Female head width	-0.1356	0.217	Non-phylogenetic	Equal	0.602
Male head width	-0.1958	0.178	Non-phylogenetic, Pure-phylogenetic	Equal	
Female thorax width	-0.0085	0.482	Non-phylogenetic, Pure-phylogenetic	Equal	0.287
Male thorax width	-0.1193	0.224	Non-phylogenetic	Equal	
Female body length	-0.1263	0.256	Punctuated, Non-phylogenetic	Equal	0.152
Male body length	-0.1146	0.342	Punctuated, Non-phylogenetic	Equal	

Genitalic morphology					
Overall left cercus shape			Pure-phylogenetic	Distance	2.25
Left cercus PC1	0.7523	0.004	Pure-phylogenetic	Distance	
Left cercus PC2	0.3741	0.02	Pure-phylogenetic	Distance	
Left cercus PC3	0.1163	0.283	Non-phylogenetic	Equal, Distance	
Overall right cercus shape			Pure-phylogenetic, Non-phylogenetic	Distance	0.32
Right cercus PC1	0.5514	0.013	Pure-phylogenetic	Distance	
Right cercus PC2	0.6999	0.002	Non-phylogenetic	Equal, Distance	
Right cercus PC3	0.1599	0.115	Non-phylogenetic	Distance	
Overall intradextral process shape			Pure-phylogenetic, Non-phylogenetic	Distance	1.277
Intradextral process PC1	0.4226	0.039	Pure-phylogenetic	Distance	
Intradextral process PC2	0.1211	0.223	Non-phylogenetic, Pure-phylogenetic	Distance	
Intradextral process PC3	0.0408	0.418	Non-phylogenetic	equal	

[1] Phylogenetic autocorrelation calculated with Phylogenetic Independence (Abouheif 1999).

[2] Best model of evolutionary change as determined by CoMET. Models presented have AIC scores of 2 or more difference from any other model, and AIC scores are within 2 where two separate models are presented. CoMET pairs models, but we separate phylogenetic signal and tempo of change results for ease of reading.

[3] Pagel's κ that best describes each character.. κ values approximating zero indicate speciational change (equal model in CoMET), while values approximating one indicate continuous change (distance model in CoMET). κ values are calculated for genitalic shapes by analyzing the distances between species in PC space, which is why only one value is given for each genitalic structure. κ could not be calculated for characters with missing species data. Pagel's κ value was calculated for all characters for which data on all ten *Timema* species. κ was not calculated for those characters where data was missing, and a negative κ value was rounded up to 0, as the implications for a negative κ do not differ from those of $\kappa = 0$.

Table 4-3: Comparison of sexual trait diversification patterns and functions in Enellagma damselflies and Timema walking-sticks.

	Enellagma	***Timema***
Mating system	Females arrive at pond to oviposit. Males of several species compete for the opportunity to mate with females, with the successful male clasping on to the female. Females may accept or reject males at this stage. (Fincke 1982, Fincke et al. 2007)	After initial contact, males and females pair with conspecifics. Males then court females, and female rejection at this stage is uncommon. Males and females copulate for several hours, after which males remain on the female mate guarding for up to several days (Arbuthnott and Crespi In Press)
Species recognition	Females reject heterospecific males at clasping (Fincke et al. 2007, McPeek et al. 2008)	Males usually do not pair with heterospecific females (Arbuthnott and Crespi In Press)
Sexual selection	Males of several species compete for control of female, males attempt to mate with any female (Fincke 1982, Fincke et al. 2007)	Males preferentially court females from the same host plant, and females can reject males during copulation attempts (Arbuthnott and Crespi In Press)
Genitalic function	Clasping, species recognition (McPeek et al. 2008)	Clasping, possible sexual selection or sexual conflict functions
Pattern of genitalic change	Proportional to speciation events (McPeek et al. 2008)	Primarily continuous
Courtship function	No courtship	In *T. cristinae* males preferentially court females from the same host plant (Arbuthnott and Crespi In Press), possible role in early reproductive isolation
Pattern of courtship change	Not relevant	Proportional to speciation events

Table 4-4: review of the phylogenetic signal of courtship.

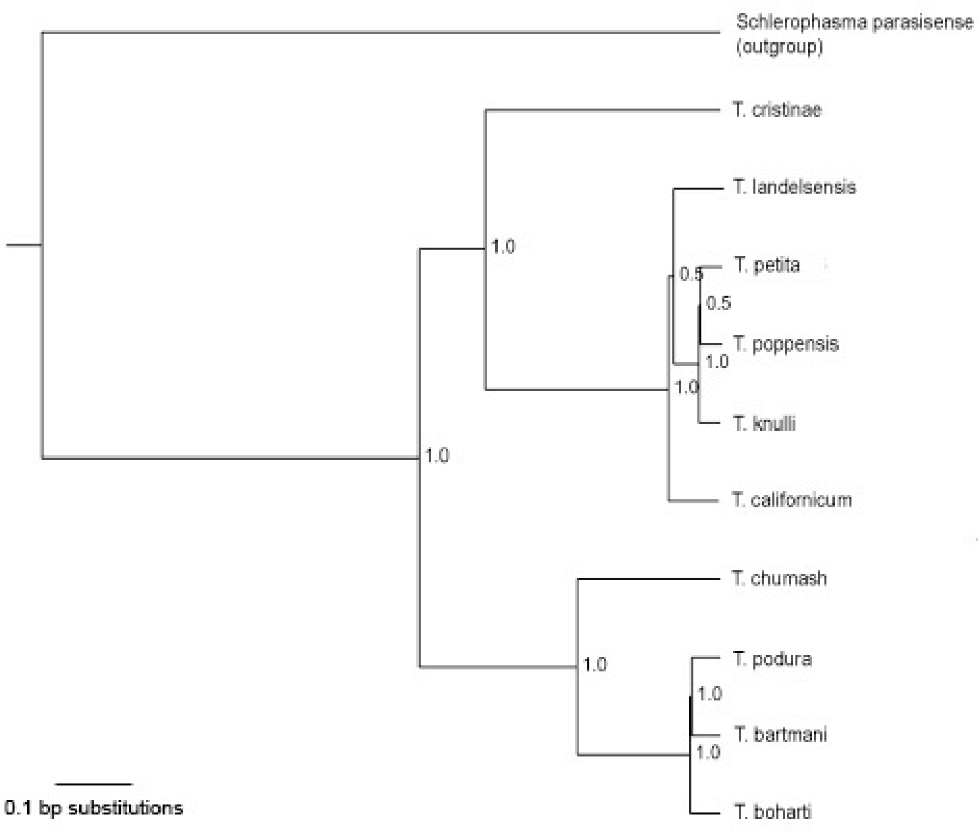
Organism	**Reference**	**Courtship trait**	**Phylogeny constructed from**	**Phylogenetic autocorrelation singal?**[1]	**Method of assessment**	**Reson for conclusion**	**notes**
Crustaceans							
Ilyoplax crabs	(Kitaura and Wada 2006)	Leg waving display	mtDNA	Y, N	Mapped courtship characters onto phylogeny	Some behaviours are clade-specific, others are not	Different behaviours have different evolutionary histories
Fiddler crabs	(Sturmbauer et al. 1996)	Courtship behaviour	mtDNA	N	Mapped courtship characters onto phylogeny	Courtship characters are not clade-specific	
Insects							
Drosophila eremophila and *anceps* groups	(Alonso-Pimentel et al. 1995)	Song	Chromosome inversion data	Y	Mapped courtship characters onto phylogeny	Closely related species have similar song structures, there is little convergence of song structure	
Drosophila	(Ferveur 2005)	Cuticular hydrocarbons	Literature review	N	Mapped cuticular hydrocarbon profiles onto phylogeny	Observable convergence in cuticular hydrocarbon structures	

Drosophila willistoni complex	(Gleason and Ritchie 1998)	Song	Period locus	N	Correlated song distance with genetic distance	Insignificant negative correlation between courtship and genetic distances, independent gains and losses of courtship elements	
	(Ritchie and Gleason 1995)	Song	Allozymes	Y	Compare song groupings with allozyme groupings	Song and allozyme groupings are qualitatively similar	
Drosophia plantibia subgroup	(Hoikkala et al. 1994)	Song	Chromosome banding	N	Mapped courtship characters onto phylogeny	Independent gains and losses of courtship characters	
Drosophila Montana subgroup	(Hoikkala et al. 2005)	Song		N	Compared interpopulation song variation with genetic distance	Song variation is independent of genetic distance	
Drosophila	(Markow and O'Grady 2005)	Visual display, song	Literature review	Y, N	Mapped courtship characters onto phylogeny	Independent gains of visual courtship, courtship song number often clade specific	Visual display does not reflect phylogeny, song reflects phylogeny only in some groups
Gryllus crickets	(Harrison and Bogdanowicz 1995)	Song	mtDNA	Y	Mapped courtship characters onto phylogeny	Song is clade-specific	

Arachnids							
Jumping spiders	(Maddison and Hedin 2003)	Visual cues	mtDNA + nDNA	Y	Compared phylogeny based on courtship to phylogeny based on genetic data	Species relationships similar for courtship and genetic phylogenies	
Amphibians							
Salamanders	(Mead and Verrell 2002)	Courtship behaviour	mtDNA	Y	Mapped courtship characters onto phylogeny	Few secondary gains or losses of courtship elements	
Tungara frogs	(Ryan and Rand 1995)	Song	Morphology + allozymes	N	Correlated song similarity with genetic distance	No significant correlation between song similarity and genetic distance	
	(Ryan et al. 1996)	Song	Allozymes	Y	Correlated song similarity with allozyme groupings	Significant correlation between song similarity and allozyme groupings	
Lizards							
Lacertid lizards	(In Den Bosch and Zandee 2001)	Courtship behaviour	mtDNA	Y	Mapped courtship characters onto both behavioural and genetic phylogenies	Behavioural and genetic phylogenies are similar, show similar patterns of character gains and losses	
Phrynosomatid lizards	(Wiens 2000)	Display behaviour	Morphology + allozymes	Y	Mapped courtship characters onto phylogeny	Courtship behaviour shows fewer independent gains and losses than morphology	

Birds							
Oropendolas birds	(Price and Lanyon 2002)	Song	mtDNA	Y	Consistency and retention index calculations, mapped courtship characters onto phylogeny	Low homoplasy for most courtship characters, positive correlation between song similarity and genetic distance	
Manakins	(Prum 1990)	Display behaviour	Syrinx morphology	Y	Compared phylogeny based on courtship and phylogeny based on morphology	Courtship and morphology based phylogenies differ in few species relationships	
	(Prum 1998)	Mechanical sounds	Syrinx morphology	N	Mapped courtship characters onto phylogeny	Independent gains and losses of mechanical sounds	
Storks	(Slikas 1998)	Display behaviour	DNA-DNA hybridization	Y	Compared phylogeny based on courtship and phylogeny based on genetic data	Behavioural and genetic phylogenies are similar	

[1] Positive or no phylogenetic signal, as determined by authors.



	Leg waving frequency	antenna waving frequency	Left cercus PC1	Right cercus PC1	Intradextral process PC1
Schlerophasma parasisense (outgroup)					
T. cristinae	3.4	1.5	0.005	0.002	-0.003
T. landelsensis	3.7	1.6	0.006	0.0008	0.001
T. petita	4.4	1.6	0.004	0.002	0.0009
T. poppensis	2.5	1.1	0.003	0.003	-0.002
T. knulli	4.6	1.0	0.004	0.001	0.0005
T. californicum	4.2	1.6	0.005	0.003	0.00003
T. chumash	3.9	2.0	-0.01	0.002	-0.002
T. podura	1.3	2.2	-0.009	-0.007	0.007
T. bartmani	3.9	2.2	-0.008	-0.004	0.004
T. boharti	5.3	1.7	-0.009	-0.002	0.003

Figure 4-1: Clocklike tree for the ten examined Timema species based on Bayesian inference.

Values at nodes are posterior Bayesian probabilities. Species averages of key courtship and genitalic characters are given alongside species relationships.

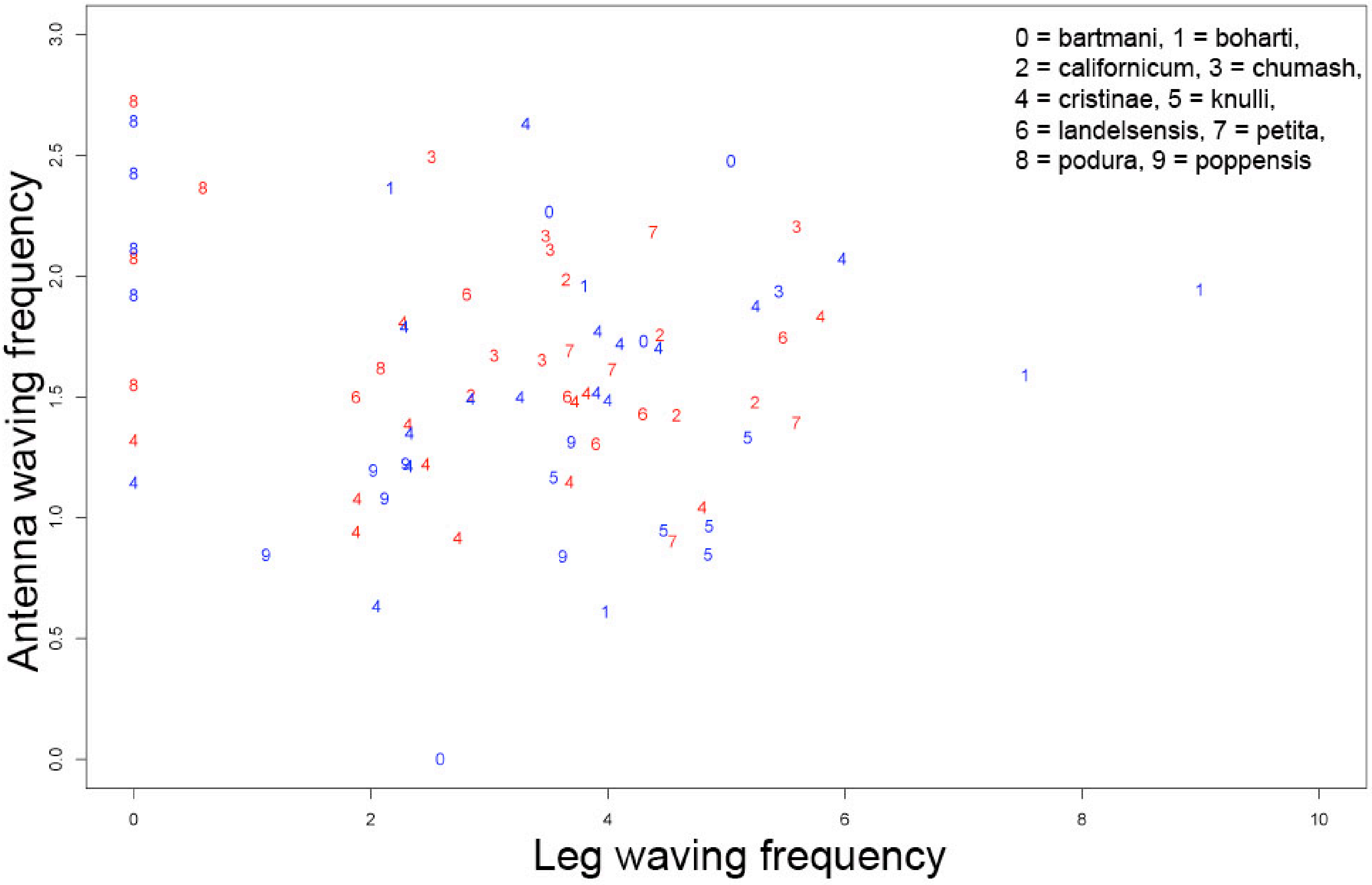


Figure 4-2: Diversity of courtship among Timema species.

Leg and antenna waving frequencies are two characters that display species-level differences. Members of different species are denoted using numbers, while colours denote host-type groupings (blue are from relatively needle leaf plants, red are from relatively broad leaf plants).

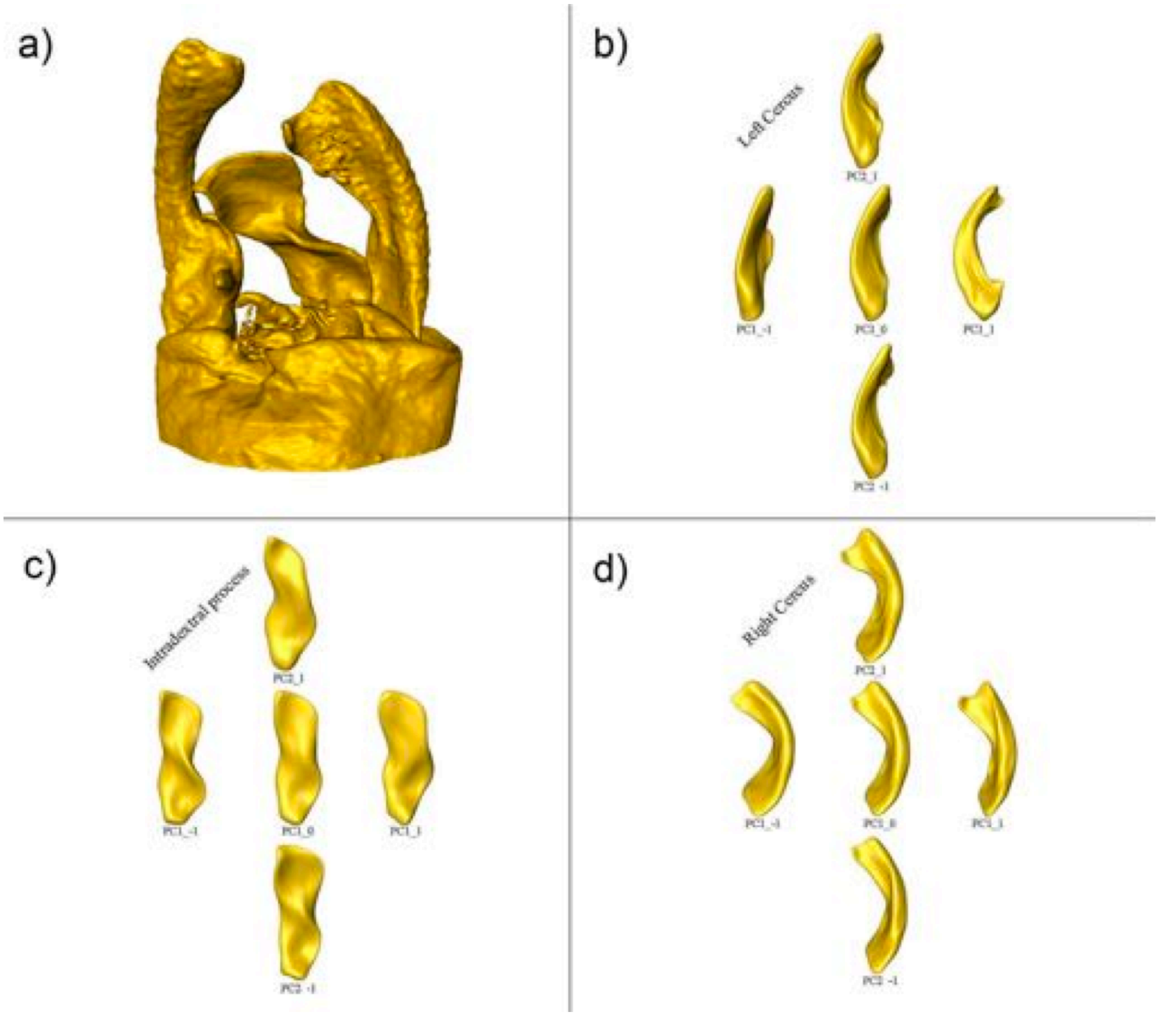


Figure 4-3: Three-dimensional representations of Timema external genitalic structures.

a) ventral view of complete external genitalia of one *T. cristinae* individual, showing (left to right) the left cercus, intradextral process, and right cercus. b - d) Changes in genitalic shape along principal component axes for *Timema* left cercus, intradextral process, and right cercus.

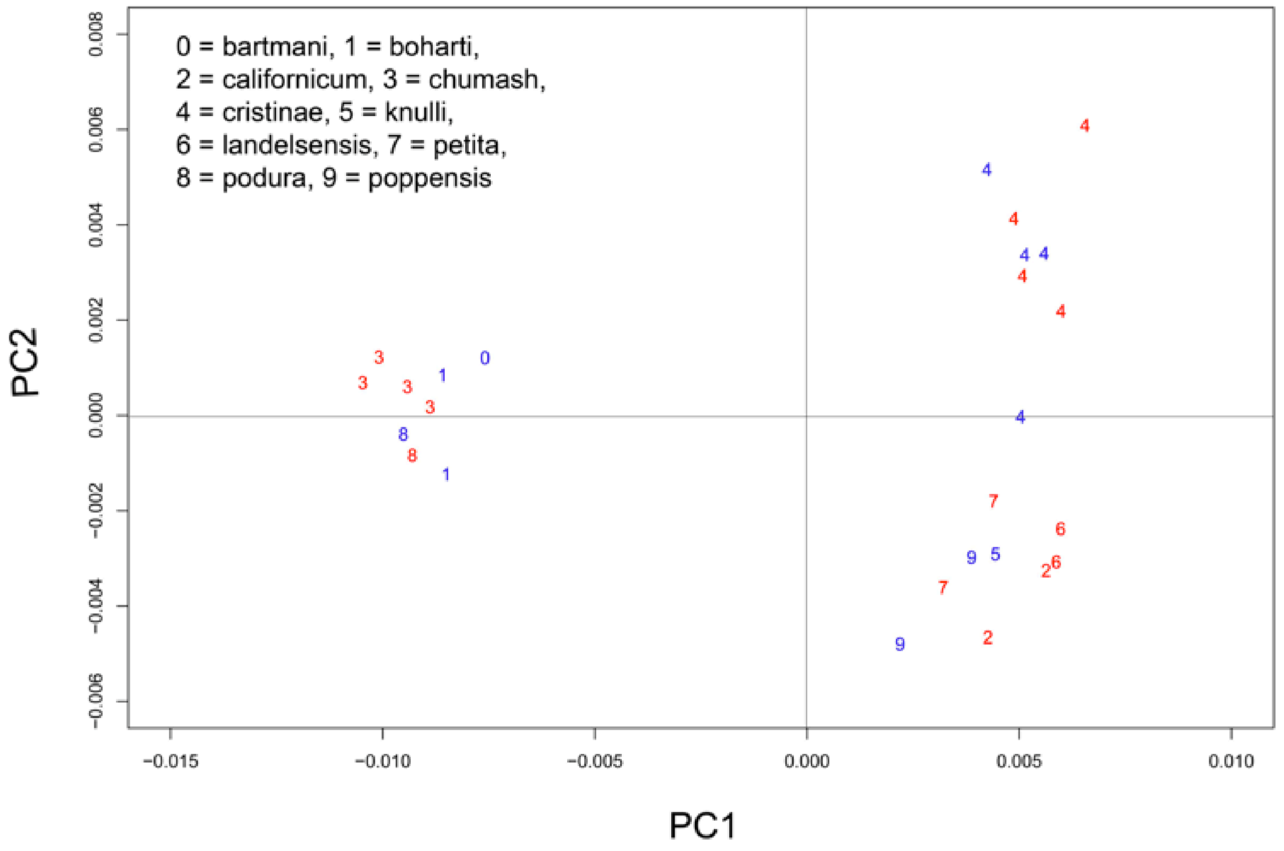


Figure 4-4: Principal component representations of left cercus morphology for all measured Timema individuals.

Genitalic shape clusters by species and clade (southern clade represents *T. bartmani*, *T. boharti*, *T. chumash*, and *T. podura*, northern clade represents all other species). Intraspecific variation can be seen in *T. cristinae*. Numbers denote species, and colours denote host-type of individuals (blue are from needle leaf plants, red are from broad leaf plants). Principal component axes correspond to those in Fig. 2.

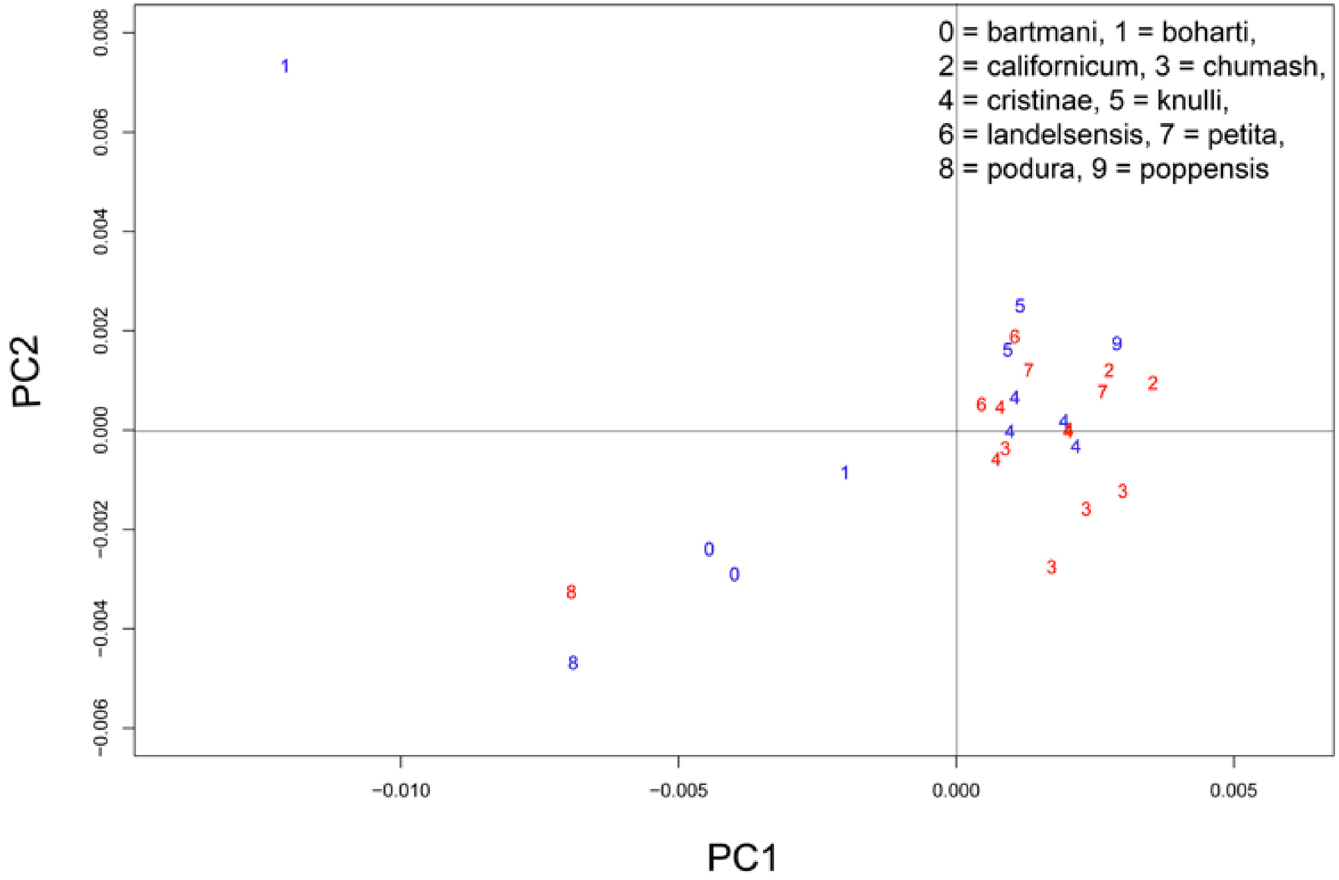


Figure 4-5: Principal component representations of right cercus morphology for all measured Timema individuals.

When the outlier boharti right cercus data is removed, all CoMET and κ results are unchanged. Numbers denote species, and colours denote host-type of individuals (blue are from needle leaf plants, red are from broad leaf plants). Principal component axes correspond to those in Fig. 2.

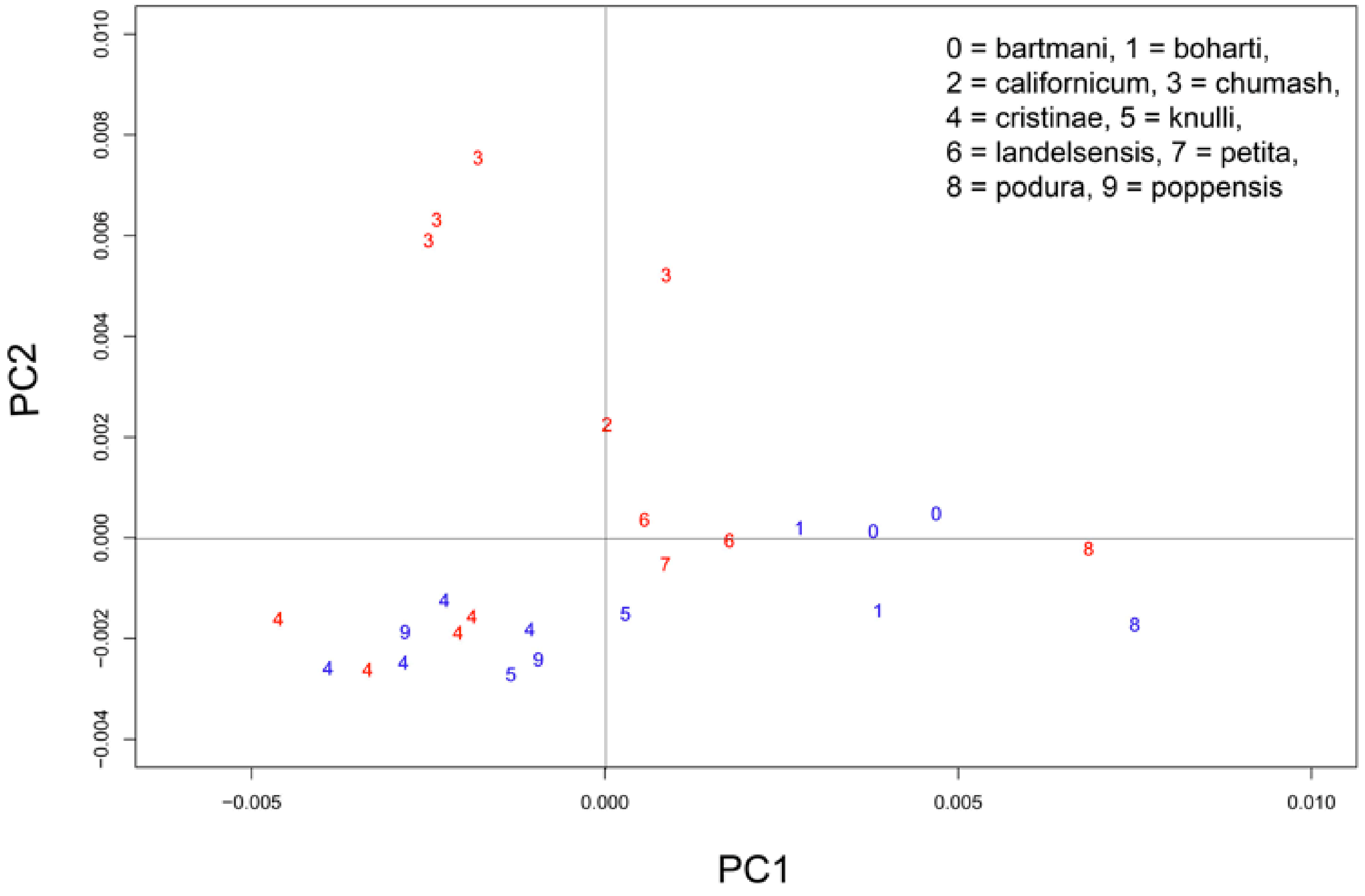


Figure 4-6: Principal component representations of intradextral process morophology for all measured Timema individuals.

Numbers denote species, and colours denote host-type of individuals (blue are from needle leaf plants, red are from broad leaf plants). Principal component axes correspond to those in Fig. 2.

CHAPTER 5: GENERAL CONCLUSIONS

In this thesis, I have examined the diversification of mating behaviour and the influence of such behaviour on the evolution of reproductive isolation. In doing so, I have sought to determine what selective forces shape the diversification of reproductive phenotypes, and how such changes may contribute to speciation. I have combined reviews of the literature with empirical research on *Timema* walking-sticks to test hypotheses on the selective pressures underlying courtship diversification and the evolution of reproductive isolation.

First, I reviewed the available literature on the genetic architecture of courtship behaviour in insects (Chapter 2). Genetic architecture underlying reproductive or adaptive traits is thought to influence the likelihood of divergence and speciation, as well as the pace of trait divergence. The majority of courtship traits for which data is available show that such traits are underlain by few loci of major effect, genetic architecture which may contribute to rapid diversification of courtship in many organisms, as well as the likelihood of speciation for those species for which courtship influences reproductive isolation. Also, by examining the genetic basis of courtship differences, I conclude that for all examined *Drosophila* species, intraspecific and interspecific variation in courtship do not represent a continuum at the genetic level. Current evidence therefore suggests that sexual selection and species-recognition do not form a continuum on a genetic level, and that stages of accumulation or replacement of reproductive barriers, rather than one barrier initiating and completing isolation, characterize speciation.

I next investigated mating behaviour in the context of reproductive isolation in *Timema* walking-sticks (Chapter 3). Within *T. cristinae*, isolation between ecologically divergent populations is manifested as decreased courtship between individuals from separate host-plants, though reproductive isolation was not always present among populations within this species. Among three species, *T. cristinae*, *T. podura*, and *T. chumash*, isolation was manifested as decreased probability of pairing between males and females of separate species. In both population and species levels of isolation, mate discrimination occurred before courtship, suggesting that differences in courtship do not contribute to reproductive isolation in *Timema* at present. Furthermore, because mate discrimination involved different behavioural processes within and among species, I conclude that separate traits and processes underlie isolation at intermediate and complete stages of divergence, which suggests that the evolution of reproductive isolation does not represent a continuous process at the behavioural level for *Timema*.

When I examined the evolutionary history of *Timema* courtship behaviour by mapping courtship characteristics onto phylogeny and determining the patterns of phenotypic change, I found that the majority of phenotypic divergence occurs during speciation or population divergence (Chapter 4). Speciational change, together with observed intraspecific variation in courtship, suggests that changes in courtship behaviour may have been important early in the evolution of reproductive isolation. Genitalic morphology of *Timema*, in contrast, appears to have changed by a continuous process, implicating sexual selection or sexual conflict as being important to genitalic diversification. Overall, separate sexual characters can differ slightly in details of divergence pattern, and characters can diverge independently of one another, but similar pressures influence characters that are functionally linked (separate courtship and genitalic morphology characters here), which subsequently promote similar patterns of divergence. When information on the evolutionary history of sexual phenotype is combined with information from current reproductive isolation in *Timema*, there is evidence for a scenario in which courtship is active in the initiation of reproductive isolation, but where subsequent strengthening of premating isolation relieves courtship of its role in isolation. As reproductive isolation evolves, courtship appears to be replaced by traits influencing the likelihood of courtship performance at intermediate stages of isolation, and subsequently by traits influencing the likelihood of pairing at nearly complete stages of isolation.

Timema premating isolation is a large component of reproductive isolation both during early stages of divergence (Nosil et al. 2002, 2003; Nosil 2004) and in maintaining species reproductive boundaries (Arbuthnott and Crespi In Press). As in some other animals (Boake and Hoikkala 1995; Price and Boake 1995; Saarikettu et al. 2005), variation in courtship behaviour does not influence current reproductive isolation in *Timema* (Arbuthnott and Crespi In Press). However, the evolutionary history of courtship diversification suggests that changes in courtship may be central to the evolution of reproductive isolation (Chapter 4).

Therefore, current reproductive isolation does not equate to past evolutionary processes.

Sexual selection and species recognition have often been thought of as two ends of a continuum of accumulating reproductive isolation, due largely to classic genetic models of speciation (Lande 1981; Kirkpatrick 1982). By such scenarios, a single trait can both initiate and complete isolation, making speciation a continuous process. However, sexual selection and species recognition have been shown to be separate processes underlain by separate traits for several organisms (Boake et al. 1997; Carracedo et al. 2000; Hankison and Morris 2002). In this thesis, there are several lines of evidence to further suggest that the evolution of reproductive isolation is a discontinuous process: first, sexually selected traits acting within species and species-recognition traits are genetically separate in all available *Drosophila* data (Arbuthnott In Press). Second, processes and traits underlying mate discrimination between ecologically divergent populations within species (intermediate reproductive isolation) differ from processes and traits underlying species-recognition (nearly complete reproductive isolation) in *Timema* (Arbuthnott and Crespi In Press). Finally, traits that are potentially important in the initiation of reproductive isolation are not equivalent to current reproductive barriers in *Timema* (Chapter 4). Therefore, there is a growing body of evidence suggesting that speciation is a discontinuous process where isolating traits accumulate or are replaced through evolutionary time. Thus, speciation likely represents several discrete steps of character change leading to increased reproductive isolation, rather than a continuous slope.

References

Arbuthnott, D. In Press. The genetic architecture of insect courtship behavior and premating isolation. Heredity.

Arbuthnott, D., and B. J. Crespi. In Press. Courtship and mate discrimination within and between species of Timema walking-sticks. Anim. Behav.

Boake, C. R. B., M. P. DeAngelis, and D. K. Andreadis. 1997. Is sexual selection and species recognition a continuum? Mating behavior of the stalk-eyed fly Drosophila heteroneura. Proc. Natl. Acad. Sci. U. S. A. 94:12442-12445.

Boake, C. R. B., and A. Hoikkala. 1995. Courtship behavior and mating success of wild-caught Drosophila-silvestris males. Anim. Behav. 49:1303-1313.

Carracedo, M. C., C. Suarez, and P. Casares. 2000. Sexual isolation between Drosophila melanogaster, D-Simulans and D-mauritiana: sex and species specific discrimination. Genetica 108:155-162.

Hankison, S. J., and M. R. Morris. 2002. Sexual selection and species recognition in the pygmy swordtail, Xiphophorus pygmaeus: conflicting preferences. Behav. Ecol. Sociobiol. 51:140-145.

Kirkpatrick, M. 1982. Sexual selection and the evolution of female choice. Evolution 36:1-12.

Lande, R. 1981. Models of speciation by sexual selection on polygenic traits. Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences 78:3721-3725.

Nosil, P. 2004. Reproductive isolation caused by visual predation on migrants between divergent environments. Proc. R. Soc. Lond. Ser. B-Biol. Sci. 271:1521-1528.

Nosil, P., B. J. Crespi, and C. P. Sandoval. 2002. Host-plant adaptation drives the parallel evolution of reproductive isolation. Nature 417:440-443.

Nosil, P., B. J. Crespi, and C. P. Sandoval. 2003. Reproductive isolation driven by the combined effects of ecological adaptation and reinforcement. Proc. R. Soc. Lond. Ser. B-Biol. Sci. 270:1911-1918.

Price, D. K., and C. R. B. Boake. 1995. Behavioral reproductive isolation in Drosophila-silvestris, D-heteroneura, and their F1 hybrids (Diptera, Drosophilidae). J. Insect Behav. 8:595-616.

Saarikettu, M., J. O. Liimatainen, and A. Hoikkala. 2005. Intraspecific variation in mating behaviour does not cause sexual isolation between Drosophila virilis strains. Anim. Behav. 70:417-426.

APPENDIX

Relevant references excluded from Chapter 2

Reference	Reason excluded
Boake et al. 1998	Measure presence/duration of courtship rather than a specific trait
Civetta and Cantor 2003	Measure latency and duration of courtship rather than a specific trait
Collins et al. 1999	No conclusion about number of loci underlying courtship trait
Krebs 1990	Measure presence of courtship rather than a specific trait
Oliveira et al. 2001	No conclusion about number of loci underlying courtship trait
Päällysaho et al. 2003	No conclusion about number of loci underlying courtship trait
Peixota et al. 2001	No conclusion about number of loci underlying courtship trait
Ptacek 2002	No conclusion about number of loci underlying courtship trait
Ritchie and Kyriacou 1996	No conclusion about number of loci underlying courtship trait
Ruedi and Hughes2008	No conclusion about number of loci underlying coutship trait
Sawamura et al. 2008	No quantification of courtship traits
Soubotcheva et al. 2004	No conclusion about number of loci underlying courtship trait

Boake CRB, Price DK, Andreadis DK (1998). Inheritance of behavioural differences between two interfertile, sympatric species, *Drosophila silvestris* and *D. heteroneura*. *Heredity* **80**: 642-650.

Civetta A, Cantor EJF (2003). The genetics of mating recognition between *Drosophila simulans* and *D. sechellia*. *Genet. Res. Camb.* **82**: 117-126.

Collins RD, Jang Y, Reinhold K, Greenfield MD (1999). Quantitative genetics of ultrasonic advertisement signaling in the lesser waxmoth *Achroia grisella* (Lepidoptera: Pyralidae). *Heredity* **83**: 644-651.

Krebs RA (1990). Courtship behavior and control of reproductive isolation in *Drosophila mojavensis*: genetic analysis of population hybrids. *Behav. Genet.* **20**: 535-543.

Oliveira SG, Bottecchia M, Bauzer LGSR, Souza NA, Ward RD, Kyriacou CP, et al. (2001). Courtship song genes and speciation in sand flies. *Mem Inst Oswaldo Cruz* **96**: 403-405.

Päällysaho S, Aspi J, Liimatainen JO, Hoikkala A (2003). Role of X chromosomal song genes in the evolution of species-specific courtship songs in *Drosophila virilis* group species. *Behav. Genet.* **33**: 25-32.

Peixoto AA, Costa R, Hall JC (2001). Molecular and behavioral analysis of sex-linked courtship song variation in a natural population of *Drosophila melanogaster*. *J. Neurogenetics* **14**: 245-256.

Ptacek MB (2002). Patterns of inheritance of mating signals in interspecific hybrids between sailfin and shortfin mollies (Poeciliidae: *Poecilia: Mollienesia*). *Genetica* **116**: 329-342.

Ritchie MG, Kyriacou CP (1996). Artificial selection for a courtship signal in *Drosophila melanogaster*. *Anim. Behav.* **52**: 603-611.

Ruedi EA, Hughes KA (2008). Natural genetic variation in complex mating behaviors of male *Drosophila melanogaster*. *Behav. Genet.* **38**: 424-436.

Sawamura K, Zhi H, Setoguchi K, Yamada H, Miyo T, Matsuda M, et al. (2008). Genetic analysis of female mating recognition between *Drosophila ananassae* and *Drosophila pallidosa*: application of interspecific mosaic genome lines. *Genetica* **133**: 179-185.

Soubotcheva EA, Romanova NI, Kim AI (2004). Study of male mating behavior in some *Drosophila melanogaster* strains in experiments with fertilized females. *Russ. J. Genet.* **40**: 732-736.