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ECOLOGY AND EVOLUTION OF THE SEXUAL AND ASEXUAL TIMEMA STICK INSECTS

Larose Chloé

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Département d'écologie et d'évolution

ECOLOGY AND EVOLUTION OF THE SEXUAL AND ASEXUAL *TIMEMA* STICK INSECTS

Thèse de doctorat ès sciences de la vie (PhD)

présentée à la

Faculté de biologie et de médecine
de l'Université de Lausanne

par

Chloé LAROSE

Biologiste diplômée du Master de l'Université de Rennes 1, France

Jury

Prof. Ron Stoop, Président
Prof. Tanja Schwander, Directeur de thèse
Prof. John Pannell, expert
Prof. Hanna Kokko, expert

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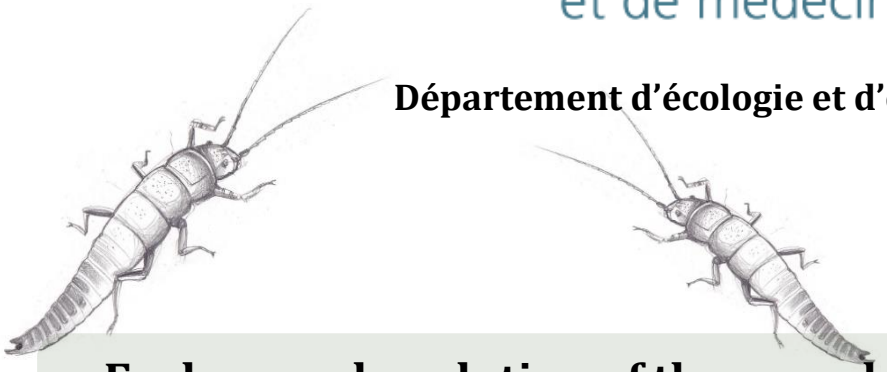

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	Madame	Prof. Hanna **Kokko**

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pour le Doyen
de la Faculté de biologie et de médecine

Prof. Ron Stoop

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Chloé.

'' Distance is a relative notion

You think you've filled the void you reached

But you finally realize

The more you dig the less you sink

The less you stop the more you start

You focus on the goal

But once you're close enough

If only everything was slightly different..

You feel like getting closer to the edge

But you don't know on which side you stand

The beginning might just be the end

A point of view could never be shared ''

I.D

A B S T R A C T

'Reproduction' is one of the key characteristic of life. Despite this, our knowledge of the evolution of reproductive systems is still incomplete. In particular, the reasons for why the vast majority of eukaryotes use sex, and thus take a complicated and costly detour to reproduction, when straightforward routes, such asexuality, are available, remains a central and largely unanswered question in evolutionary biology. The aim of my thesis is to contribute to the understanding of this evolutionary mystery, and for that I use stick insects of the genus *Timema* as a study system. This small group of herbivorous insects, endemic to Western United States is ideal for studying and comparing sexual and asexual reproduction as seven asexual lineages have been identified in this group, each with a sexual sister species, allowing us to make multiple independent comparisons between sexual and asexual lineages.

The perhaps most broadly accepted theoretical argument is that sex allows selection to work efficiently, which would ultimately favor the adaptive potential of populations. My objective during this thesis was to test two theories directly related to this, but working each time in two successive steps: i) I started by clarifying the ecological and evolutionary aspects and mecanisms concerned by these theories in *Timema* focusing only on sexual species and thus independently of the reproductive mode, ii) and I then empirically tested these theories. Specifically, I first investigated whether sexuals are able to exploit more ecological niches than asexuals, which would give them an advantage in fluctuating or heterogeneous environments. From this first investigation, I overall found that sexual species are systematically using a larger portion of their environment than their asexual relatives, but I did not find this pattern regarding their intrinsic and physiologic abilities to use their environment. The reduced portion used by asexuals is thus likely a consequence of external and biotic interactions that affect asexuals more strongly than sexuals. I secondly aimed to empirically test if sex confer an advantage when the allele combinations that are favored by selection vary over time, as it is the case in context of coevolution with parasites. My work suggests that parasites are indeed contributing to the maintenance of sex in *Timema*. In the last part of the thesis, I finally present some preliminary results regarding new *Timema* populations that I discovered by chance, that feature unusual reproductive strategies with a mixture of sexual, asexual ad facultatively asexual individuals. These populations will be very profitable for future research concerning the evolution of reproduction in *Timema*. Overall this thesis work contributes to a better understanding of several aspects of the ecology and evolution of *Timema* stick insects in partiular, and more generally contribute to give novel insights in the understanding of the maintenance of sex in the living world.

RESUMÉ FRANÇAIS

L'une des caractéristiques essentielles d'un être vivant est sa capacité à se reproduire. Malgré cela, notre connaissance et compréhension de l'évolution de la reproduction est encore très partielle. En particulier, les raisons pour lesquelles la grande majorité des eucaryotes utilise un mode de reproduction aussi compliqué et raffiné que le sexe, alors que des manières beaucoup plus simples de se reproduire existent reste une véritable enigme de la biologie évolutive. Le but de ma thèse est de contribuer à la résolution de ce mystère évolutif. Pour cela j'étudie les phasmes du genre *Timema*, un petit groupe d'insectes herbivores endémique de l'ouest des États-Unis. C'est un système d'étude idéal pour comparer les coûts et bénéfices de la reproduction sexuée et de la reproduction asexuée car sept lignées asexuées ont été identifiées au sein de ce groupe, chacune avec une espèce ancestrale soeur sexuée. Cela nous permet de faire des comparaisons indépendantes entre lignées sexuées et asexuées.

L'un des arguments théoriques le plus largement proposé pour expliquer la prédominance du sexe, est qu'il permet à la sélection naturelle de fonctionner plus efficacement, ce qui favoriserait le potentiel adaptatif des populations. Au cours de cette thèse, j'avais pour objectif de tester deux théories s'incrivant dans ce contexte. J'ai travaillé en deux étapes successives : J'ai tout d'abord étudié et clarifié les aspects et processus écologiques et évolutifs concernés par ces théories chez les *Timema* en se concentrant exclusivement sur les expèces sexuées, et donc indépendemment du mode de reproduction, puis, dans un second temps, j'ai testé empiriquement ces théories. Premièrement, j'ai verifié si les sexués sont capables d'exploiter plus de niches écologiques que les asexués, ce qui leur confererait un avantage au sein des environnements fluctuants ou hétérogènes. J'ai trouvé que les espèces sexuées utilisent systématiquement une plus large portion de leur environnement que les espèces asexuées, mais je n'ai pas retrouvé un tel pattern en ce qui concerne leurs capacités intrinsèques et physiologiques à utiliser cet environnement. Cette utilisation de l'environnement réduite des asexués comparé aux sexués indique que les pressions externes et biotiques affectent plus fortement la capacité des asexués à exploiter leur environnement que celle des sexués. Deuxiemement, j'ai verifié empiriquement si le sexe confère un avantage lorsque les combinaisons d'allèles favorisées par la sélection varient au cours du temps, comme c'est le cas lors d'une coevolution hotes-parasites. Mon travail suggère que les pressions parasitaires contribuent effectivement au maintien du sexe chez leurs hotes *Timema*. Dans la dernière partie de cette thèse, je présente des résultats préliminaires concernant de nouvelles populations de *Timema* que j'ai découvert par chance au cours du doctorat. Ces populations ont des stratégies reproductives inhabituelles comprenant une mixture d'individus sexués et asexués, et seront très utiles lors des futures recherches concernant l'évolution de la reproduction. Dans l'ensemble, ma thèse contribue à une meilleure connaissance de l'écologie et de l'évolution des phasmes *Timema*, et contribue plus généralement à comprendre pourquoi le sexe est le mode de reproduction prédominant au sein du monde vivant.

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- GENERAL INTRODUCTION -

GENERAL INTRODUCTION

One of the few key characteristics defining a living being is its ability to reproduce. Reproduction is the way in which genetic information is transmitted and thus structured from one generation to the other (De Meeûs *et al.* 2007). The different ways to reproduce have therefore different consequences on the potential of diversification and adaptation of populations (Maynard Smith 1978) and ultimately influence the evolution and structuration of biodiversity. However, despite its essential role, our understanding of the evolution of reproductive systems is still incomplete and full of questions.

The most widespread mode of reproduction is sex. One definition of sex posits that it corresponds to the fusion of a female gamete with a male gamete, with gametes produced via meiosis. The offspring's genome is then a combination of the mother and the father. However, even though the vast majority of organisms are reproducing via sexual reproduction, there are many other ways to reproduce, such as different forms of asexual reproduction. Contrary to sexual reproduction, asexual reproduction is the production of offspring by a single parent. Several kinds of reproduction are referred to as asexuality; though in this thesis, I consider only female-producing parthenogenesis, which corresponds to the formation of an embryo from an unreduced egg cell by a virgin female individual.

The paradox of sex: theoretical costs and benefits of sexual and asexual reproduction

Sexual reproduction occurs throughout the tree of life, in the majority of lineages, and has been maintained since several billion years (Miyamoto & Fitch 1996; Gu 1997; Goodenough & Heitman 2014; Speijer *et al.* 2015; Speijer 2016). However, potential advantages of other forms of reproduction including asexuality, seem to exceed those of sex (see review by Lehtonen *et al.* 2012). To date, this evolutionary paradox is still the subject of numerous scientific debates and reflections (e.g., Howard & Lively 1994; Barton & Charlesworth 1998; West *et al.* 1999; Hamilton 2001; Kondrashov 2001; Rice &

Chippindale 2001; Otto 2009; Sharp & Otto 2016; Burke & Bonduriansky 2017; Neiman *et al.* 2017).

One of the most obvious benefits of asexuality is that it is theoretically associated with an immediate **two-fold demographic advantage** compared to species that invest equally in both sexes (formalized by Williams, 1975 and Maynard Smith 1978). Since only females can directly produce offspring, asexual reproduction avoids the cost of males inherent to sexual reproduction (Fig. 1). Moreover, asexuality provides **reproductive insurance**. Indeed, contrary to sex, only one parent is required to produce offspring; asexuality therefore avoids the risk of not finding a mating partner which would lead to reproductive failure (e.g., Lively 1992; Johnson 1994; Hörandl 2006; Schwander *et al.* 2010). Asexuality also **avoids some potentially risky behaviors** associated with sex. For example, sex often requires finding and attracting mates and eventually mating (Landolt 1997), which may bear direct costs and generate increased risks of predation (e.g., Sakaluk 1990) or infection with sexually transmittable diseases (e.g., Thrall *et al.* 1997). Nevertheless, asexuality is rare while obligate sex is widespread. Therefore, to explain the ubiquity of sex, it is necessary to understand how sex can generate benefits that are substantial enough to fully compensate the costs it generates.

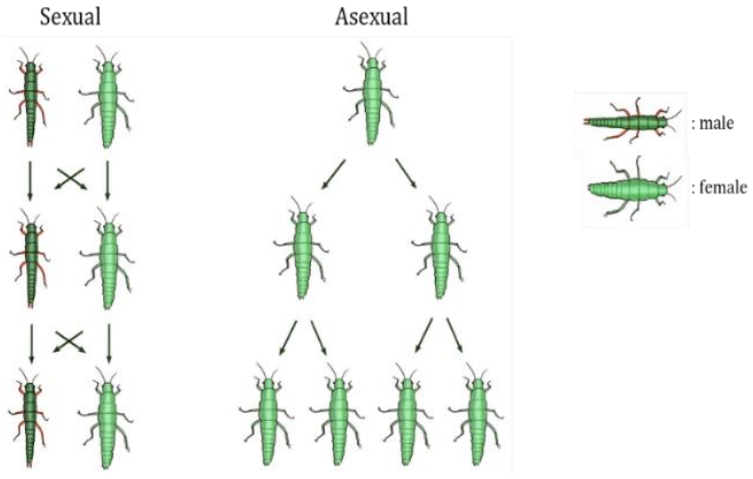


Figure 1. A schematic illustration of the twofold cost of males.

Over 40 different theories, encompassing genetic and ecological arguments, propose mechanisms through which sex can generate benefits, both in the short and in the long term. The perhaps most broadly accepted view is that sex allows selection to work efficiently. A consequence of efficient selection is that **sexual reproduction would facilitate the purging of mildly deleterious mutations over time** which may gradually accumulate in finite asexual populations, in a ratchet-like manner (Muller 1964; Lynch *et al.* 1993; West *et al.* 1999). This argument is largely supported by empirical evidence for increased accumulation of deleterious mutations in genomic regions with low levels of recombination and in asexual animal lineages (Normark & Moran 2000; Bachtrog *et al.* 2004; Paland & Lynch 2006; Neiman *et al.* 2010; Henry *et al.* 2012). It is believed that mutation accumulation plays an important role in limiting the persistence of asexual lineages in the long term, contributing to the confinement of asexuals to the terminal branches of phylogenetic trees. In other words, the current asexual lineages would be mostly recent on the evolutionary timescale (< 1 million years; Bell 1982; Lynch *et al.* 1993; Rice 2002; Neiman *et al.* 2009; Schwander & Crespi 2009b). Efficient selection also **favors the adaptive potential of populations** (Goddard *et al.* 2005; Goddard 2016). Indeed, sex breaks linkage disequilibria among loci. If some loci have different fitness effects, breaking the associations among loci would increase the variance in fitness under some conditions. It will thereby provide a benefit by increasing the response to selection in the next generation (Hill & Robertson 1966; Kondrashov 1988; Barton & Charlesworth 1998; Colegrave 2002; Otto & Lenormand 2002).

A context where rapid responses to selection would provide strong benefits is coevolution between hosts and parasites. The "*Parasite hypothesis for sex*" (Hamilton 1980; Bell 1982; Seger & Hamilton 1988; Ladle 1992; Lively 1996, 2010) is in fact currently considered as one of the most likely for explaining the maintenance of sex in natural population. Sex, and recombination, could be advantageous when the allele combinations that are favored by selection vary over time. This would be especially likely in a context of co-evolutionary interactions between species, such as coevolution between hosts and parasites. Parasites would be under selection to infect the most common host genotype (e.g., Dybdahl & Lively 1995; Jokela *et al.* 2009; Paczesniak *et al.* 2014). By inducing negative frequency-dependent selection (Neiman & Koskella 2009; Leung *et al.* 2012; Vergara *et al.* 2014),

conditions would be more likely to favor sex over asexuality as it facilitates the production of variable and rare host genotypes over generations (Hamilton 1980; Hamilton *et al.* 1990). By contrast, genetically uniform clonal lineages would be more vulnerable to parasitism over time.

Because sex can generate offspring with rare and novel gene combinations, sex could also be advantageous in heterogeneous and saturated environments (e.g., Becks & Agrawal 2010). By contrast, once an asexual lineage is generated, because it stems from a sexual population, it will only contain a small portion of the total genetic variability of this sexual population. As a consequence, the phenotypic distribution of a new, recently derived clone is expected to be narrower than that of its genetically variable sexual ancestor, as predicted by the "*Frozen niche variation model*" (Vrijenhoek 1984; Case & Taper 1986; Weeks 1993; Vrijenhoek & Parker Jr 2009). Sexual populations could therefore exploit more ecological niches than asexual lineages. Although the *FNV* is the most intuitive and popular theory predicting the consequences of asexuality on individual and population niche breadth, there are other theories that challenge it. This is the case of the *General-purpose genotype* (i.e., GPG) theory which proposes that individual clones should have broader environmental tolerances than their sexual relatives (Lynch 1984). Indeed, they suggest that a temporally and/or a spatially variable environment should favor clones with a broad environmental tolerance and therefore select the genotypes characterized by low variance in fitness across environments. This may lead to asexual populations with broader ecological niches than the sexual counterparts. These two theories are both supported by some empirical examples (i.e,, FNV: Gray and Weeks, 2001, vs GPG: Weider, 1993; Van Doninck et al., 2002). To date, the few existing empirical examples do not allow us to clearly understand and conclude about the consequences of sex and asexuality on the breadth of the ecological niche, neither at the individual level, nor at the level of the lineages, populations or species.

In addition to costs and benefits of sexual reproduction, the frequency of sexual and asexual reproduction may also depend on how easily they can evolve (see Engelstädter 2008). In this context, asexuality may be rare, at least in metazoans, partly because it is the derived reproductive mode (sexual reproduction is the ancestral state of all

metazoans). It may thus just be difficult to evolve asexuality from a sexual ancestor. To date, to disentangle the paradox of sex, it is therefore crucial to evaluate precisely the costs and benefits of these two reproductive systems, and especially the advantages of sex, but also to determine the mechanisms and processes underlying the evolutionary transitions from sexual reproduction to asexuality. It is in this theoretical context and at this state of the current research that my thesis work is taking place.

The Timema *genus: an ideal group to adress the paradox of sex*

An ideal study group for comparing sexuals and asexuals is *Timema*. In *Timema* seven independently derived asexual lineages, each with a closely related and apparently ecologically similar sexual counterpart, have been identified (Law & Crespi 2002a, b; Schwander & Crespi 2009a; Fig. 2). This allows to perform replicated comparisons between sexual and asexual lineages. To date, only obligate sexuals and obligate asexuals are known in this group, facultative parthenogens have never been described except some evidence for tychoparthenogenesis (i.e., rare and spontaneous hatching of a small proportion (< 1%) of the unfertilized eggs in sexual species; Schwander *et al.* 2010). In more, no overlap between sexual and asexual population ranges from a given sister species pair has been observed in this group (Fig. 3). Moreover, because the asexual *Timema* lineages vary in age (Law & Crespi 2002a; Schwander *et al.* 2011; Bast *et al.* 2018), it is further possible to assess the possible consequences of asexuality over a range from recently derived to long-term asexuality. One of the lineages (*T. genevievae*, see Fig.2) is even so ancient that it is one of the few organisms currently considered as a "scandal of evolution" (see Judson & Normark 1996).

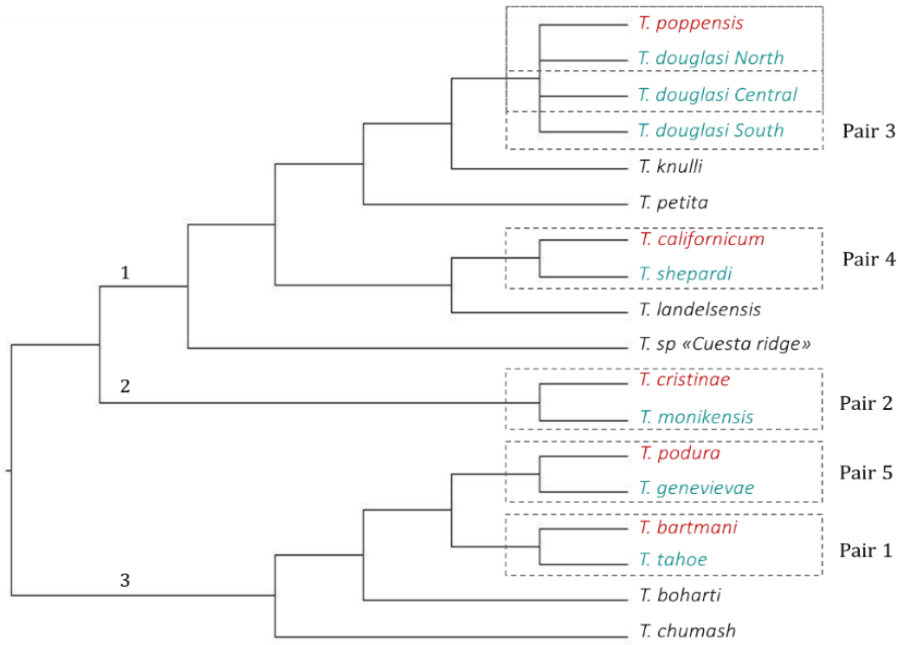


Figure 2. *Timema* phylogeny highlighting the sexual and asexual species pairs. Phylogeny redrawn from (Riesch *et al.* 2017), with the seven asexual lineages added from Schwander *et al.* (2011). Their five sexual sister species, that we used for studies in his thesis, are written in red. Numbers 1, 2 and 3 correspond to the three described *Timema* clades, respectively Northern, Santa Barbara, and Southern clade. Throughout this thesis I will number the sexual-asexual species pairs as shown on this figure.

Timema is a small genus of stick insects considered as the sister group of other phasmids (Terry & Whiting 2005; Zompro 2005). It originated about 30 million years ago in southern California or Arizona, in conjunction with the origin and spread of the chaparral biome to which most species are adapted (Axelrod 1980, 1989; Sandoval *et al.* 1998; Riesch *et al.* 2017). It is found throughout California (Fig. 3) and consists of 23 known species of wingless herbivorous stick insects (Phasmatodea: Timematidae) feeding mostly on the leaves of trees and shrubs from a range of very diverse host plants comprising both angiosperms and conifers. The name Phasmatodea comes from Ancient Greek "φάσμα" (phasma), meaning “an apparition” or “phantom”. This name comes from the fact that phasmids are mimics of their natural background and especially of their host plant (Fig. 4) which also constitute their habitat. They rest on branches or leaves of vegetation during the day and feed at night, relying on crypsis for protection against predators (Sandoval 1994a, b). Their color patterns are matching the color patterns of their host plants, and several species exhibit host-associated color polymorphisms (Sandoval 1994a, b).

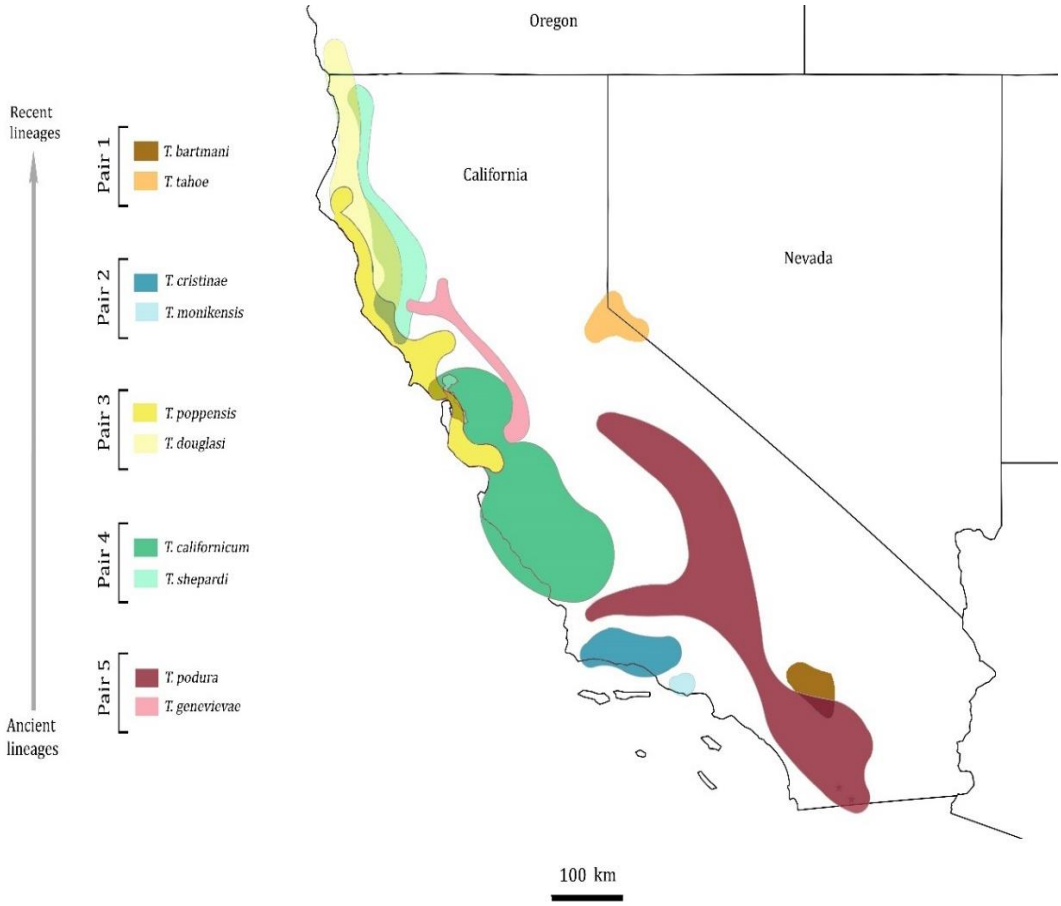


Figure 3. Approximal geographical distributions of ten described *Timema* species. The represented species correspond to the five sister pairs of sexual and asexual *Timema* species used as model systems in this thesis. The dark and clear shadings are used to respectively distinguish sexual and asexual species ranges. We used all known GPS coordinates, that we implemented in QGIS2.14.21 to estimate species ranges.

Figure 4. Pictures of *Timema* stick insects. *T. douglasi* (A, D), *T. chumash* (B) and *T. poppensis* (C) camoufling in their respective host plants douglas fir (A, D), oak (B) and redwood (C).

Figure 5. Illustration of *Timema monikensis* with color polymorphism. Here we show four adult females. *Pictures from Bart Zijlstra*

Thesis work: How I addressed the paradox of sex using Timema

The first step of my thesis research is to characterize in detail several aspects of the biology and ecology of *Timema* stick insects, the ultimate goals being to test several hypotheses suggested to help explain the maintenance of sex. I started with the study of the evolution of specialization in this group, aiming to compare niche breadths in sexuals and asexuals. In 2000 and 2002, phylogenetic and ecological analyses from Crespi and Sandoval suggested a trend for *Timema* to increase their level of ecological specialization over the course of evolution, while acquiring the ability to use novel plants and thus during the colonization of California state. However, these studies, like most studies that discuss the evolution of specialization (Colwell & Futuyma 1971; Nyffeler & Sterling 1994; Blüthgen *et al.* 2006; Slatyer *et al.* 2013; Forister *et al.* 2014; Rasmann *et al.* 2014), are based only on the realized feeding niche, and thus the number of host plant species or families they used in nature. The fundamental niche of insects, i.e. the full range of diets on which they would be able to survive, grow and reproduce in the absence of predation and competition, has never been studied. In **Chapter I**, I aimed to characterize realized and fundamental feeding niche breadths of *Timema* species, to determine i) whether *Timema* performance on host plants is constrained by trade-offs between alternative plants, ii) whether the fundamental feeding niche breadth changes following a shift to a novel host, iii) whether *Timema* stick insects retain the ability to use ancestral host plants following host shifts, and finally iv) if fundamental and realized feeding niche sizes are correlated.

Overall, a review of literature completed with personal observations allowed me to establish an exhaustive list of the host plants used by each of the 23 known *Timema* stick insects' species in the wild and thus to estimates their realized feeding niche. I also performed feeding experiments using a subset of the known *Timema* host plants, in order to estimate the fundamental feeding niche of a number of *Timema* species. Finally, Chapter I also explored potential mechanisms that could generate variable performances of insects on different plant species, by analyzing phenolic and terpenic plant secondary metabolites, known as toxins and/or feeding deterrents for many herbivorous insects (Bi & Felton 1995; Wink 1998; Acamovic & Brooker 2005; Dearing et al. 2005; Fürstenberg-Hägg et al. 2013). Surprisingly, I found that realized and fundamental feeding niche breadths are not at all correlated in this genus but that, on the contrary, the most generalist *Timema* species use a single or very few plant species in the wild. I overall found that *Timema* lineages retain the ability to use ancestral host plants after shifting to new plants. More generally, if such adaptations to new host plants can sometimes enlarge the fundamental niche, this could facilitate future host shifts in the same lineage, which could in turn drive frequent host turnovers via positive feedback mechanisms. This study thus provides interesting new insights into the evolutionary dynamics of host use and host range in herbivorous insects in general.

This first study allowed me to better understand the processes underlying the evolutionary history of host plant specialization in *Timema*. Thanks to this refined knowledge, I was then able to compare, in each sexual-asexual *Timema* sister species pair, the realized and the fundamental feeding niches and to discuss the results in relation to the theories currently proposed regarding niches in sexual and asexual organisms (i.e., The *Frozen Niche Variation* model and the *General purpose Genotype* hypothesis, see above). These aspects of my work are presented in **Chapter II**. Overall, I did not find a general pattern regarding niche sizes of sexuals and asexuals. Instead I found a different pattern according to the age of the *Timema* asexuals. For the most recent asexual tested (from the *T. cristinae*/*T. monikensis* species pair; Fig. 2), the asexual is significantly more specialist than the sexuals as predicted by the FNV model, whereas for the oldest *Timema* asexual (from the species pair *T. podura*/*T. genevievae*; Fig. 2), the asexual is significantly more generalist than the sexual, as predicted by the GPG theory. This result is likely to be

of interest regarding the age of the ancient asexual *T. genevievae*. Indeed, the GPG hypothesis could explain how such an ancient asexual lineage, considered as a mystery or even a scandal of evolution, could be maintained for so long.

In the second part of this thesis, I aimed to test another major hypothesis raised to explain the maintenance of sex, *the parasite hypothesis for sex*. However, prior to my thesis, very little was known about parasites infecting *Timema* in nature. I therefore started by conducting a study to better understand the interactions between *Timema* and their parasites. One of the outputs of this work is presented in **Chapter III**. In this third chapter, I report and identify an endoparasitic nematode infecting different *Timema* species, which induces dramatic effects on their fitness (the host is killed). Given the direct exposure of the endoparasites to the host's immune system in the haemolymph, and the consequences of infection on host fitness, I predicted that divergence among hosts could invoke parallel divergence in the endoparasites. However, I found a complete lack of co-divergence between the endoparasitic nematodes and their hosts in spite of extensive genetic variation among hosts and among parasites. Instead, there was strong isolation by distance among the endoparasitic nematodes, indicating that geography plays a more important role than host-related adaptations in driving parasite diversification in this system. This project, in addition to improving our knowledge about the interactions between stick insects and their parasites, has allowed us to contribute more broadly to the research field that is host-parasite evolution and coevolution. Indeed, it comes in addition to the accumulating evidence for lack of co-diversification between parasites and their hosts at macro-evolutionary scales, which contrasts with the overwhelming evidence for co-evolution within populations. It therefore calls for studies linking micro- vs macro-evolutionary dynamics in host-parasite interactions.

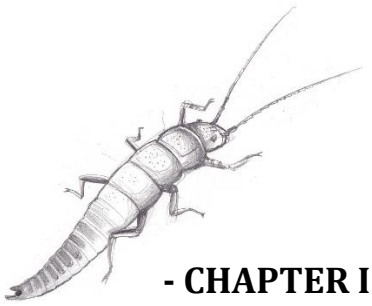
This first parasite candidate, given its very low prevalence in *Timema* natural populations and this complete lack of co-divergence with its host was a bad candidate to test the *Parasite hypothesis for sex* in *Timema*. I then identified a fungal parasite that infects a large proportion of individuals from at least seven different *Timema* species in their natural habitat. I experimentally found that it triggers the host's immune system, negatively affects host fitness and can be transmitted between *Timema* individuals. I quantified the

prevalence of fungal infections in natural populations of sexual and asexual *Timema* species from four sexual-asexual sister species pairs, and I found that it was congruent with the *Parasite hypothesis of sex*. I finally found evidence suggesting local adaptation between *Timema* hosts and their fungal parasites. In combination, these experiments provide the first study that investigates the *Parasite hypothesis for sex* by conducting replicated sexual-asexual lineages comparisons. I found that these fungal parasites may generate selection for sexual reproduction in *Timema* walking stick insects. These aspects of my work are presented in **Chapter IV**.

Finally, in **Chapter V**, I examined two geographic areas previously considered as inhabited by the obligate parthenogen *T. douglasi* (Fig. 3). In these areas, *Timema* are living along two corridors of several kilometers and are interesting because non-negligible proportions of males were found among the females in natural populations. In order to precisely characterize these populations, I sampled two transects recording precise sex-ratios along both corridors. In the first transect I found that 100% female spots alternated with spots containing 9 to 26% males. This suggests: i) mixed populations of sexual and asexual individuals co-occurring, ii) facultative parthenogens, or iii) high production of accidental males. Along the second transect 50:50 sex-ratio locations are followed at only a few meters distance by 100:0 sex-ratio locations, which suggest for the first time in this group an overlap or a very close proximity between a sexual and an asexual population. For each sampling location of both transects, I isolated numerous juvenile individuals, and I studied both the hatching timing and hatching success of eggs laid by virgin and mated females and the mating behavior of virgin females. The results indicate the existence of mixed populations where obligate sexuals and asexuals co-occur, as well as the existence of facultative parthenogens in the *Timema* genus. It will now be possible to use *Timema* to study the evolution and the short-term costs and benefits of both reproductive systems when they are co-occurring under natural conditions.

In summary, the chapters of my thesis report a series of investigations done with the ultimate goal of contributing to understand the maintenance of sex. It begins with the study of the evolution of specialization in the *Timema* genus (**Chapter I**) which gave novel

insights for comparing the fundamental and realized niche breadth of sexuals and asexuals in *Timema* (**Chapter II**). I intended to understand what maintained sex in this genus and instead found a possible explanation to understand why asexuality was maintained for so long time in this group. I therefore aimed to test another hypothesis, *the parasite hypothesis for sex*, and started by the identification of several parasites. I studied the evolutionary interaction of both an endoparasite nematode and a fungal parasite with *Timema* (**Chapters III and IV**) and found some insights suggesting that parasites may contribute to the maintenance of sex in this genus (**Chapter IV**). I finally characterized new *Timema* populations with interesting reproductive systems which make the *Timema* group interesting to investigate the evolution, causes and consequences of sexual and asexual reproduction both in the short and in the long term (**Chapter V**). In each chapter, I present a more detailed theoretical context to the hypotheses tested and discuss their wider implications. Overall this thesis work contributes to a better understanding of several aspects of the biology and ecology of *Timema* stick insects, and more generally of herbivorous insects, and contribute to our understanding of the maintenance and predominance of sex in the living world, but also the persistence of one of the very rare species having lived without sex for so long (i.e. *Timema genevievae*).



- CHAPTER I -

Evolutionary dynamics of specialization in herbivorous stick insects

Running head:
Evolutionary dynamics of specialization

C. Larose, S. Rasmann and T. Schwander

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Author Contributions: CL and TS designed the study. SR provided methods, materials, labspace and performed the metabolomics analysis. CL and TS collected all other data and CL analysed the results. All co-authors contributed to the manuscript.

ABSTRACT

Factors explaining herbivorous insect mega-diversity include the ability to shift and quickly adapt to different host plant species. However, how fundamental feeding niches change and contribute to the realized host ranges following adaptation remains to be addressed when studying the radiation of an insect group. We conducted feeding experiments in a phylogenetic framework using *Timema* stick insects, which range from specialist to generalist species. We found that ecological specialists (occurring on a single or very few plant species in natural populations) retained plasticity in host plant use and, surprisingly, featured broader fundamental feeding niches than ecological generalists. In line with classical theory, we found that specialization at the fundamental niche level comes at a cost of reduced ability to use non-native host plants. Conversely, species with a generalist niche show little to no trade-offs in performance across multiple alternative host plants. Generalist fundamental feeding niches and ecological specialization jointly evolved in species that shifted from angiosperm to conifer hosts, which are chemically very different. Such fundamental feeding niche expansions following host shifts could facilitate future host shifts in the same lineage, which could in turn drive frequent host turnovers via positive feedback mechanisms. By coupling metabolomics with analyzes of the fundamental and realized feeding niches of multiple species, our study provides novel insights into the evolutionary dynamics of host range expansion and contraction in herbivorous insects.

Keywords: Host shift, Host use, Plant-insect interaction, Realized versus fundamental niche, Specialization, *Timema* stick insect, Trade-off

INTRODUCTION

Long standing hypotheses suggest that the evolution of the tremendous diversity of insect herbivores (Lawton 1983; Strong *et al.* 1984; Mitter *et al.* 1988; Farrell 1998; Novotny *et al.* 2006) relates to speciation driven by adaptation to novel host plants (Mitter *et al.* 1988; Dyer *et al.* 2007; Futuyma & Agrawal 2009). Many studies have focused on identifying the genetic basis of adaptations to novel hosts (Via 1991b; Sezer & Butlin 1998; Feder *et al.* 2003; Nosil 2007; Soria-Carrasco *et al.* 2014; Simon *et al.* 2015), but why or how novel host plants can be colonized at first remains largely unknown (Mayhew 2007; Winkler & Mitter 2008; Janz 2011). Indeed, multiple constraints such as plant species-specific chemical compounds, which reduce insect growth and survival, are expected to hamper the ability of insect herbivores to shift to novel hosts (Scriber 1984; Hartley & Jones 1997; War *et al.* 2013a, b; Portman *et al.* 2015).

Overcoming constraints imposed by plant chemical compounds should be especially difficult for insect species that are ecologically specialized, which is the case for the vast majority of herbivorous insects (e.g., Fox & Morrow 1981; Scott 1986; Janzen 1988; Thompson 1994). Indeed, approximately 76% of all herbivorous insects are estimated to be mono- or oligophagous, feeding on plant species belonging to a single genus or family in a given location (Forister *et al.* 2014). A frequently evoked explanation for herbivore specialization is the existence of trade-offs; where adaptation to the chemistry of one host plant negatively influences the ability to circumvent the chemistry of another host (Gould 1979; Futuyma & Moreno 1988; Schultz 1988; Jaenike 1990; Fry 1996, 2003; Kuwajima *et al.* 2010; Agrawal 2011; Rasmann & Agrawal 2011; Forister *et al.* 2012). However, empirical examples illustrating such trade-offs remain scarce (Karban 1989; Fry 1990;

Via 1991a; MacKenzie 1996; Agrawal 2000) and many studies testing for trade-offs find no evidence supporting their existence (Thompson 1996; Abrahamson & Weis 1997; Agosta & Klemens 2009; Bernays & Graham 2014; Gompert *et al.* 2015).

We hypothesized that the presence or absence of trade-offs, or, more generally, the ability to use different plant species as hosts, is influenced by the evolutionary history of an insect lineage (see also Futuyma & McCafferty 1990). Specifically, if insect lineages can retain the ability to use their ancestral hosts as a food source after having shifted to a novel host, there may be little or no trade-off. On the other hand, if highly specialized lineages cannot retain the ability to use their ancestral hosts, strong trade-off in host use are expected. The vast majority of comparative studies on herbivore specialization so far have focused on the number of hosts used in natural population (i.e. the *realized* feeding niche) (Colwell & Futuyma 1971; Futuyma & McCafferty 1990; Nyffeler & Sterling 1994; Blüthgen *et al.* 2006; Slatyer *et al.* 2013; Rasmann *et al.* 2014; Fordyce *et al.* 2016). Therefore, studies need to evaluate the ability to use different hosts in a phylogenetic context (Janz *et al.* 2001), by comparing the realized feeding niche with the ability for colonizing novel hosts (i.e. the *fundamental* feeding niche as defined as the potential range of plants allowing the insect to survive, grow and reproduce; Whittaker *et al.* 1973; Leibold 1995).

Here, we used *Timema,* a genus of herbivorous stick insects from western North America (Vickery 1993), and asked whether i) insect performance on host plants is constrained by trade-offs between alternative plants, ii) the fundamental feeding niche breadth changes following a shift to a novel host, iii) insects retain the ability to use ancestral host plants following host shifts, and iv) fundamental and realized feeding niche sizes are correlated.

The *Timema* genus is suited for addressing these questions because different species have colonized plants from phylogenetically distant families, ranging from one to eight families of host plants per *Timema* species (Table 1). In terms of realized feeding niche, the *Timema* genus thus comprises a range of relatively specialist to generalist species.

The *Timema* genus originated about 30 million years ago (Riesch *et al.* 2017), in conjunction with the origin and spread of the chaparral biome to which most species are adapted (Sandoval *et al.* 1998; Crespi & Sandoval 2000). Ancestral *Timema* populations were most likely associated with angiosperms characterizing the chaparral biome, specifically the genera *Ceanothus* (lilac) and *Adenostoma* (chamise) (Sandoval *et al.* 1998; Crespi & Sandoval 2000). Nonetheless, transitions from angiosperm to conifer hosts have occurred multiple times in the genus. Ten of the 23 known *Timema* species regularly use conifers from one or multiple families as hosts (Table 1). At least two conifer species (redwood, *Sequoia sempervirens* and white fir, *Abies concolor*) represent recent host shifts, as both redwood and white fir are hosts for monophyletic groups of closely related *Timema* species (Fig. 1).

To characterize the realized feeding niches for the 23 known *Timema* species, we first generated a complete list of host plants for each species, using information from previous studies and field surveys. We then estimated the breadth of the fundamental feeding niche for nine of the 23 *Timema* species, including populations from two different host plants for three of the nine species (12 populations in total). To this end, we measured juvenile insect performance on seven phylogenetically diverse plants from the *Timema* host plant species pool (Table 1). This sampling strategy allowed us to compare the breadth of the fundamental with the realized feeding niche and to test for specialization-driven trade-

offs in host use. Finally, in order to explore potential mechanisms generating variable performances of insects on different plant species, we analyze phenolic and terpenic secondary metabolites, which are toxins and/or feeding deterrents for many herbivorous insects (Bi & Felton 1995; Wink 1998; Acamovic & Brooker 2005; Dearing *et al.* 2005; Fürstenberg-Hägg *et al.* 2013).

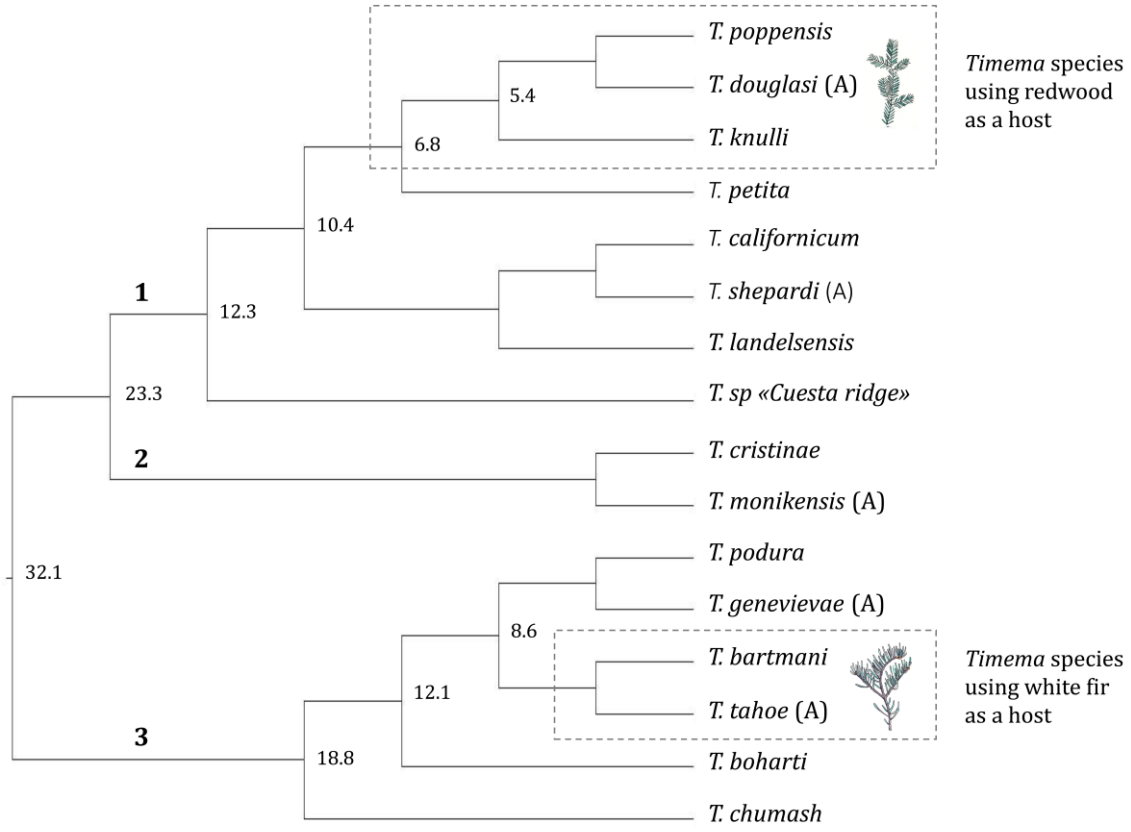
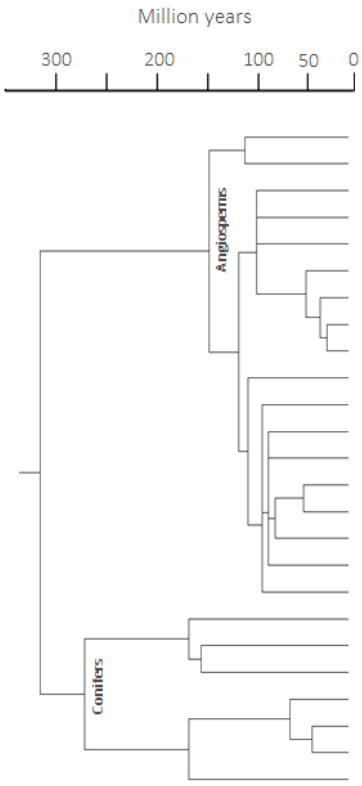


Figure 1. *Timema* phylogeny highlighting the species using the novel host plants redwood and white fir. Phylogeny redrawn from (Riesch *et al.* 2017), with asexual lineages (A) added from (Schwander *et al.* 2011). The phylogenetic position for the missing *Timema* species (see Table 1) is not known. Numbers 1, 2 and 3 correspond to the three described *Timema* clades, respectively Northern, Santa Barbara, and Southern clade.

Table 1. *Timema* species and their recorded host plants in the wild. Plants labeled with an "X" correspond to a common host for a given *Timema* species, where experimental evidence confirms that the plant is used as a food source. Plants labeled with "." correspond to rare/anecdotal observations where it is unclear whether these plants are used as a food source (or solely for resting). Columns highlighted in gray indicate the *Timema* species used in the present study, sampling locations are specified in Fig. S1. Plants used for feeding experiments are written in bold. The plants on which the corresponding *Timema* populations were collected for this study are encircled. Note two of the *Timema* species are undescribed: *Timema* 'Limberpine', mentioned first by (Sandoval & Crespi 2008), and *Timema* 'Cuesta ridge' from (Riesch *et al.* 2017). The phylogenetic distances between the plant genera are estimated with information from the public database TIMETREE. (http://timetree.org/; Hedges *et al.* 2015; Kumar *et al.* 2017)



				Northern clade								Santa Barbara clade		Southern clade						Others						
HOST PLANTS				*T. californicum*	*T. douglasi A*	*T. knulli*	*T. landelsensis*	*T. petita*	*T. poppensis*	*T. shepardi A*	*T. sp «Cuesta ridge»*	*T. cristinae*	*T. monikensis A*	*T. bartmani*	*T. boharti*	*T. genevievae A*	*T. podura*	*T. tahoe A*	*T. chumash*	*T. sp «Limberpine»*	*T. coffmani*	*T. dorotheae*	*T. maorongensis*	*T. nakipa*	*T. ritensis*	*T. nevadense*
Plant abbreviation	Common name	Latin name	Plant family																							
ely	wheatgrass	*Elymus spp*	Poaceae												•											
yuc	yucca	*Yucca spp*	Asparagaceae												X		•									
buc	buckwheat	*Eriogonum fasciculatum*	Polygonaceae												•		•					X				
mz	**manzanita**	***Arctostaphylos spp***	**Ericaceae**	(X)	•		X		•	X	X	•		•	•		X		•					X		
bal	mountain balm	*Eriodictyon spp*	Boraginaceae	•													•									
tri	American trixis	*Trixis californica*	Asteraceae														•									
coy	coyote brush	*Baccharis pilularis*	Asteraceae	•																						
bri	shrubby brickellbush	*Brickellia frutescens*	Asteraceae	•											•											
eri	eriophyllum	*Eriophyllum sp*	Asteraceae	•																						
ace	bigleaf maple	*Acer macrophyllum*	Aceraceae	•																						
oak	**oak**	***Quercus spp***	**Fagaceae**	(X)	•	•	•		•	•	X	•	•	•	•	•	X		X				X	•		
rha	spiny redberry	*Rhamnus spp*	Rhamnaceae									•														
lil	**californian lilac**	***Ceanothus spp***	**Rhamnaceae**	X	•	(X)	X	(X)	•	X	X	(X)	X		X	•	X		X			X		X		
toy	toyon	*Heteromeles arbutifolia*	Rosaceae	X						•		•							X							
pru	bitter-berry	*Prunus virginiana*	Rosaceae														•									
cha	**chamise**	***Adenostoma fasciculatum***	**Rosaceae**	X							X	(X)	X		X	X	(X)		X			•		X		
cer	mountain mahogany	*Cercocarpus betuloides*	Rosaceae	X						•		X	X		(X)	•	X		(X)							
pick	pickeringia	*Pickeringia montana*	Fabaceae									•														
wf	**white fir**	***Abies spp***	**Pinaceae**											(X)			•	X								
pin	limber pine, knobcone pine	*Pinus spp*	Pinaceae	•		X								X	•		•			X						X
df	**douglas fir**	***Pseudotsuga menziesii***	**Pinaceae**	•	X	•			X	•																
ced	Insense cedar	*Calocedrus decurrens*	Cupressaceae											•			•									
cyp	sargent cypress	*Hesperocyparis sargentii*	Cupressaceae			•					X															
jun	Juniper	*Juniperus spp*	Cupressaceae																	X					X	X
rdw	**redwood**	***Sequoia sempervirens***	**Cupressaceae**		X	(X)			(X)																	
Number of typical host plants				**6**	**2**	**3**	**2**	**1**	**2**	**2**	**5**	**3**	**3**	**2**	**4**	**1**	**5**	**1**	**5**	**1**	**1**	**1**	**1**	**4**	**1**	**2**
Number of putative* host plants				13	5	6	3	1	5	6	5	8	4	5	10	4	13	1	6	1	1	2	1	4	2	2

METHODS

Realized feeding niches

In order to characterize the breadth of the realized feeding niche at the species level, we established a list of the host plants for each of the 23 known *Timema* stick insects species from the literature (Vickery 1993; Vickery & Sandoval 1997, 1999, 2001; Crespi & Sandoval 2000; Law & Crespi 2002b; Sandoval & Crespi 2008; Riesch *et al.* 2017), and completed the list with personal observations (Table 1). In addition, we estimated the realized feeding niche at the population level for 22 populations of 9 species. To this end, we surveyed all the plants known to be used by *Timema* and we calculated the frequency of occurrence of stick insects on these different plants. (Table S1).

Fundamental feeding niches

To measure insect performance on different hosts and their fundamental feeding niche breadths, we chose seven plants known to be commonly used by several *Timema* species, while trying to cover the phylogenetic diversity of all potential host plants (Fig. 1; Table 1). Stick insects for our experiments were collected from twelve populations belonging to nine *Timema* species (Table S2) throughout California (Fig. S1) using sweep nets. We only used fourth-instar juvenile females in order to minimize age-related effects, and to avoid the spurious effects of high mortality when manipulating younger instars. Between 10 and 80 females per host plant were used to measure survival and weight gain over 10 days, for a total of 70-220 females per population (1330 insects in total; see Fig. S2 for details on the experimental set-up). The large variation in numbers of insects per population was generated by the natural variation in the availability of forth instar females in different populations, as well as by the high mortality on certain plants that

prevented us from obtaining weight gain estimates for all *Timema* populations. Whenever possible, we used more females for combinations generating high mortality.

Phylogenetic analyses for evaluating trade-offs in host use

We first tested whether closely related *Timema* species had similar performances (survival and weight gain) on the different plants. Branches from the most recent *Timema* phylogeny (Riesch *et al.* 2017) were pruned to create a phylogeny of the 12 populations from the nine species sampled for this study (Fig. 1). We used Mesquite 2.75 (Maddison & Maddison 2017) to reconstruct the ancestral states of the *Timema* performances on each of the seven plants (Mesquite module "Continuous-character Model Evaluation for phylogenetic signal testing"). Maximum parsimony with unordered, equal-weighted characters, and a cost of any state change = 1 was used to minimize the total number of character-state changes over the tree. We then compared the number of character-state changes inferred on the observed *Timema* phylogeny to the number of changes inferred on 1000 trees for which the characters were randomized across the tips in Mesquite. The null hypothesis that the character is randomly distributed on the phylogeny was rejected if the observed number of state changes fell outside of the upper or lower 5 percentiles of the random distribution (Maddison & Slatkin 1991).

We then tested for trade-offs between adaptations to defenses of alternative hosts. Such trade-offs are revealed when insect performance decreases with increasing phylogenetic distance from the native host plant species (because phylogenetically disant hosts should differ more extensively in defenses than related hosts; e.g. Rasmann & Agrawal 2011). To test this prediction, we estimated the slopes of a linear regression between the weight

gain of the insects on the seven plants and the phylogenetic distance of these plants from the native host for each of the 12 *Timema* populations.

Estimations of the degree of specialization

To quantify the breadth of *Timema* feeding niches, we calculated the Tau specialization index (τ) (Yanai *et al.* 2004), as follows:

$$\tau = \frac{\sum_{i=1}^{n}(1-\widehat{x_i})}{n-1};\ \widehat{x_i} = \frac{x_i}{\max\limits_{1\leq i\leq n}(x_i)}.$$

Where **n** corresponds to the number of plants, $\mathbf{x_i}$ represents the frequency of occurrence (for the realized niche) or the weight gain (for the fundamental niche) on plant i, and **max ($\mathbf{x_i}$)** is the maximum occurrence or weight gain for the focal population. The index ranges from 0 (generalist) to 1 (pure specialist). We chose this measure to estimate the degree of specialization because of its robustness to small sample sizes and because our data were quantitative and continuous (Kryuchkova-Mostacci & Robinson-rechavi 2016). However, this index needs positive values to be calculated. We therefore transformed percentages of weight gain, which are negative when individuals lose weight, to relative weights of insects at the end of the feeding trials (i.e., an insect that lost 30% of its weight during the trial would be assigned the value 0.7, while one that gained 30% would be assigned 1.3). Note that we used 1-Tau in Figures 2 and S5 (where specialist = 0 and generalist = 1) for ease of interpretation. To test whether broad fundamental feeding niches translate into broad realized niches at the species or population level, we correlated the specialization index Tau with the number or frequency of host plants used in natural populations (the realized feeding niche). We used Phylogenetic generalized least squares (PGLS) analyses to account for phylogenetic non-independence among

Timema species. These analyses were conducted using the *ape* (Paradis *et al.* 2004) and *nlme* (Pinheiro *et al.* 2009) R packages (R Core Team 2017) using a Brownian motion model for trait evolution.

Finally, we assessed whether increased levels of specialization translate into more pronounced trade-offs for adaptations to alternative host plants by correlating the slopes of the relationship between insect performance and phylogenetic distances of plants (as described above), using PGLS-corrected correlation.

Plant chemical profile characterization

We extracted and quantified compounds in the phenolic and terpene classes of secondary metabolites from leaves of the seven plant species included in our experiments (i.e., lil, cha, oak, mz, df, wf, rdw; see Table 1), using methods adapted from Pratt *et al.* (2014) and Moreira *et al.* (2015). For each plant species, we extracted compounds from five independent replicates for both phenols and terpenes (see detailed methods for plant chemical analyses in Appendix S1).

To ordinate the chemical diversity data found across species, we conducted a principal component analysis (PCA) based on correlation matrices using the *FactoMineR* package in R (Husson *et al.* 2008). We tested whether plants have significantly different chemical compositions by estimating the chemical variation within and between species with a permutational multivariate analysis of variance (PERMANOVA) using 10.000 permutations with the *adonis* function (Anderson 2001) implemented in the R package *vegan* (Oksanen *et al.* 2007). We then tested for a correlation between the plant species

phylogenetic distances and the chemical distances across the seven species tested using Mantel-tests with 10'000 permutations.

Finally, for the subset of chemical compounds that are present in multiple plants, we evaluated whether insect performances were negatively (or positively) correlated with the amount of a given compound. We conducted Spearman correlation tests (separately for each *Timema* population) between insect weight gain and each of the chemical compounds. These tests provided us for each *Timema* species with a list of chemical compounds significantly correlated to insect performance. We then tested whether these lists were more similar between different *Timema* populations than expected by chance, using hypergeometric tests with the *phyper* function in R (Johnson *et al.* 2005). Thus, we were not interested in the specific lists of significant chemical compounds per *Timema* population (which comprise many false positives due to multiple testing), but we were interested to see if the same compounds affect the performance of multiple *Timema* populations.

RESULTS

Insect performances on different plants

The performance (survival and weight gain during 10 days) of *Timema* individuals was strongly dependent on the plant species tested. For ten of the twelve *Timema* populations, both survival and weight gain varied significantly among individuals reared on different plants, while for the two remaining populations, only weight gain varied significantly (Table S2, Fig. S3). Insect survival and weight gain were also significantly correlated (Fig. S4; Spearman rank correlation, $r= 0.66$, $p < 0.0001$), even though the most extreme situation (i.e., when all *Timema* of a given population died on a specific host plant before 10 days) could not be included in the analysis.

Generally, we found that insect performance was not maximal on the host plant they were collected on (henceforth referred to as the native host plant) (Table S2, Fig. S3). Indeed, for only five out of the 12 populations, individuals survived best on their native host plant, while for only six out of 12 populations they gained the most weight. In some cases, the performance of insects increased dramatically when individuals were reared on plant species they never use as host in the field. For example, 100% of *T. bartmani* survived for 10 days on lilac, while only 35.4% of them survived on their native host plant, white fir (Table S2).

We also observed that some host plant species are a consistently better food source than others. For instance, lilac was almost always the best food source, even for *Timema* species that never use lilac in natural conditions. Specifically, relative survival on lilac was high for all populations (between 76.9% and 100%, Table S2), and individuals from nine

of the twelve *Timema* populations gained more weight when reared on lilac than when reared on any other plant species (Fig. S3). Lilac is the native host for only three of these nine populations (*T. cristinae*–lil, *T. knulli*-lil and *T. petita*), the six remaining ones were collected on manzanita (*T. californicum*-mz), chamise (*T. cristinae*-cha), oak (*T. californicum*–oak), mountain mahogany (*T. boharti* and *T. chumash*) or redwood (*T. knulli*-rdw). Only *T. podura*, *T. poppensis* and *T. bartmani* individuals had the highest weight gain when fed with their native host plant, with lilac ranking second.

Redwood was on the opposite end of the host plant quality spectrum, as it was only exploitable by *Timema* individuals originally collected on it. Relative survival on redwood for individuals from the two native redwood populations was high (75.0 and 86.7% for *T. poppensis* and *T. knulli*-rdw respectively; Table S1), while survival was low for all other *Timema* populations (ranging from 0% to 55.6%; Table S1). Similarily, *T. poppensis* and *T. knulli*-rdw were the only species that gained significant weight when fed with redwood for ten days (mean weight gain was 45.3% and 67.7% for the two species, respectively; Fig. S3). For the ten other populations, if individuals are able to survive for ten days on redwood, they typically lost weight (80% of surviving individuals) or only gained very little (20% of surviving individuals gained weight, with a maximum gain of 9.9%; Fig. S3). For the *T. bartmani*, *T. boharti*, *T. podura*, and *T. cristinae*-cha populations, not a single individual survived for ten days on redwood.

We observed the same pattern for *T. knulli*, the only *Timema* species using both redwood and lilac under natural conditions (Table 1). All individuals collected on redwood were able to live and grow on all tested plants (Table S2, Fig. S3). By contrast, practically all

individuals of the same species collected on lilac died or lost significant weight on redwood (Table S2, Fig. S3).

Phylogenetic trade-offs in host use

Phylogenetic analyses showed that *Timema* individuals native to angiosperms performed worst when reared on phylogenetically distant host plants, but this was not the case for individuals native to conifers (Fig. S5). For eight of the nine *Timema* populations native to angiosperms, insect performance significantly decreased, or tended to decrease, on plants as a function of increased phylogenetic distance from their native host plants, despite the small sample sizes (p varying between 0.013 and 0.075; Fig. S5). By contrast, we found no significant associations between insect performance and plant phylogenetic distance for the three populations collected on conifers (p varying between 0.12 and 0.39; Fig. S5).

Degree of specialization

Surprisingly, the fundamental and realized feeding niche breadths were not correlated, neither at the species or population level. At the species level, we found no significant correlation when considering the total number of host plants per *Timema* species (correlation corrected with Phylogenetic Generalized Least Squares (PGLS); r= 0.41, p= 0.43), or when considering only the typical plants (PGLS; r= 0.17, p= 0.75)). At the population level, we also found no correlation between Tau indices estimating the fundamental feeding niche and Tau indices estimating the realized niche (Pearson correlation test, r=0.02, p=0.91).

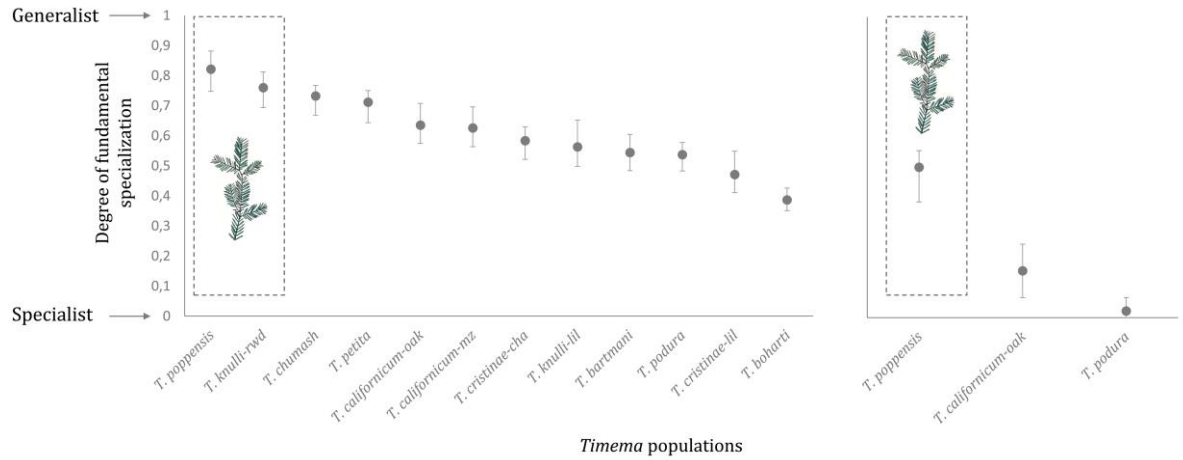


Figure 2. Breadth of the fundamental feeding niche of insect herbivores. Niche breadth is quantified via the specificity index Tau (with 95% CI), based on insect weight gain on different plants. The insect populations are listed from the least to the most specialist. Two independent analyses of specificity are presented. In the first one (A), the degree of specialization of twelve populations is based on their performance on seven plants from the *Timema* host plant pool. In the second one (B), the degree of specialization of a subset of populations is based on their performance on three novel plants not used by *Timema* stick insects in natural populations (sugar sumac, coyote bush and sage bush). The dotted rectangles surround populations native to redwood.

The specialization indices showed that the two *Timema* populations from redwood were the most generalist (dotted rectangles in Fig. 2A). The *T. knulli* population collected on redwood is also significantly more generalist (Tau = 0.23, 95% CI 0.19-0.30) than the population of the same species collected on lilac (Tau = 0.44, 95% CI 0.34-0.50). Hence, *Timema* native to redwood had a broader potential feeding niche than populations living on other host plants. In order to verify that this tendency was not only generated by the performance of the insects on redwood, we recalculated the Tau indices across six plants, excluding data from redwood. *T. poppensis*. *T. knulli*-rdw remained the most generalist species when the Tau indices were calculated without data from redwood (Fig. S6), and the Tau indices with and without redwood were strongly correlated (Pearson correlation; r: 0.96, $p < 0.0001$), indicating that the pattern was not solely driven by redwood.

These results suggest that the fundamental feeding niche of *T. poppensis* and *T. knulli*-rdw has expanded as a result of adaptation to redwood. To corroborate these findings, we reared individuals from three *Timema* populations (*T. poppensis*, *T. californicum*-oak and *T. podura*) on plants not used as hosts by natural *Timema* populations (sugar sumac, coyote bush and sage bush). Again, *T. poppensis* native to redwood performed better on these novel host plants than the two other insect species (Fig. 2B).

Finally, in line with assumptions, *Timema* populations with a narrower fundamental feeding niche (i.e. specialist populations) were more strongly constrained by trade-offs between alternative host plants than generalist populations. This was revealed by the significant positive correlation between the Tau specialization indices and the slopes of the relationship between *Timema* performance and the phylogenetic distance of plants to the native host plant (Fig. 3, PGLS; r: -0.78, p= 0.025). In other words, the more specialized a *Timema* population is, the lower is its ability to feed on distantly related host plants.

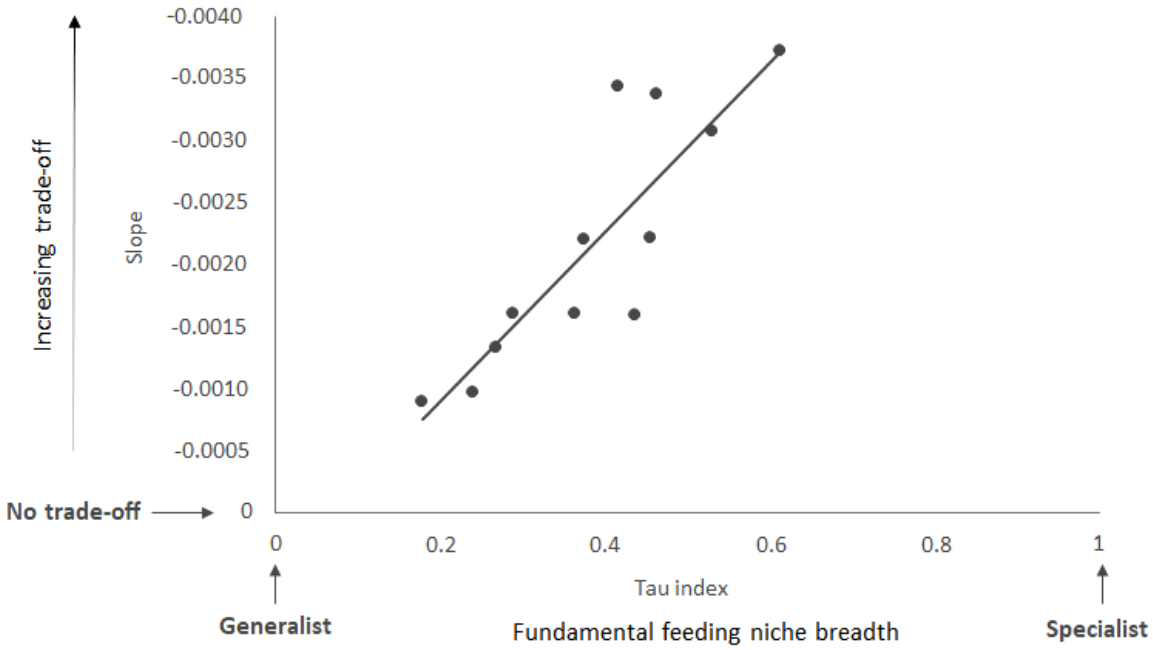


Figure 3. Feeding niche breadth affects trade-off levels for the use of alternative host plants. Each point corresponds to a *Timema* population. The trade-off level among plants is quantified by the slope of the weight gain dependent on phylogenetic distances between native host plant and food plant species for each *Timema* population (see main text). Fundamental feeding niche breadth is estimated using the Tau index.

Effect of plant chemical composition on Timema performances

To explore potential mechanisms generating variation in food quality among host plants, we studied the phenolic and terpenic secondary metabolites. We found a total of 521 different chemical compounds (28 phenols and 493 terpenes) across the seven plant species tested, with 84% of the variance explained by differences between species (PERMANOVA: $F_{6,28} = 24.5$, $p < 0.001$). In addition to chemical diversity, we also found that the total volume of compounds varied widely among plant species (volume measured as µg Gallic Acid Equivalent /g Dry Matter; average: 564µg/g; range 298 -1192), with a smaller volume in angiosperms (average: 310µg/g; range 298 – 331) than conifers (average: 902 µg/g; range 650 – 1192; Welch Two Sample t-test; $t_2 = -3.75$; p= 0.063).

The PCA differentiated four plant groups, containing: 1) lilac, 2) oak, chamise, and manzanita, 3) redwood and douglas fir, and 4) white fir (Fig. S7). Distances between terpenic compositions of plants were correlated with the between plant phylogenetic distances (Mantel-test with 10.000 permutations, r = 0.77, p= 0.014), while there was no significant correlation for the phenolic compositions (Mantel-test with 10.000 permutations, r = -0.04, p= 0.47).

Most of the isolated terpenic and phenolic compounds were specific to a single plant or a subset of plants (Fig. S8). Specifically, 45.9% of the 521 compounds were detected only in a single plant, and only 1.5% of the compounds occurred in all seven plant species (Fig. S8). To test whether the performances of multiple *Timema* species were related to similar plant chemistries, we used the 162 compounds (31%) that occurred in at least three plant species. Among these, 84 (65 after FDR = 0.05 correction) were significantly correlated to insect weight gain in at least one *Timema* population. No single compound was found to be significantly correlated with the performance of *Timema* individuals collected from both angiosperms and conifers (Fig. 4). By contrast, 26 compounds (30.5%) were significantly correlated to the weight gain of insects from six of the nine populations living on angiosperms. One additional compound was further correlated to the weight gain of individuals of both populations collected from redwood (*T. poppensis* and *T. knulli-rdw*; Fig. 4). As phenols and terpenes are known to play an important role in plant defense against herbivorous insects, these compounds were expected to negatively affect insect performances. However, 59.2% of the compounds showed a positive effect (r varying between 0.77 and 0.99; Fig. 4), suggesting that some phenolic and terpenic compounds may favor rather than constrain *Timema* performance. The number of compounds

significantly correlated to insect performance and shared among several populations significantly exceeded the amount of sharing expected by chance (Hypergeometric tests, p varying between 1e-06 and 1e-18).

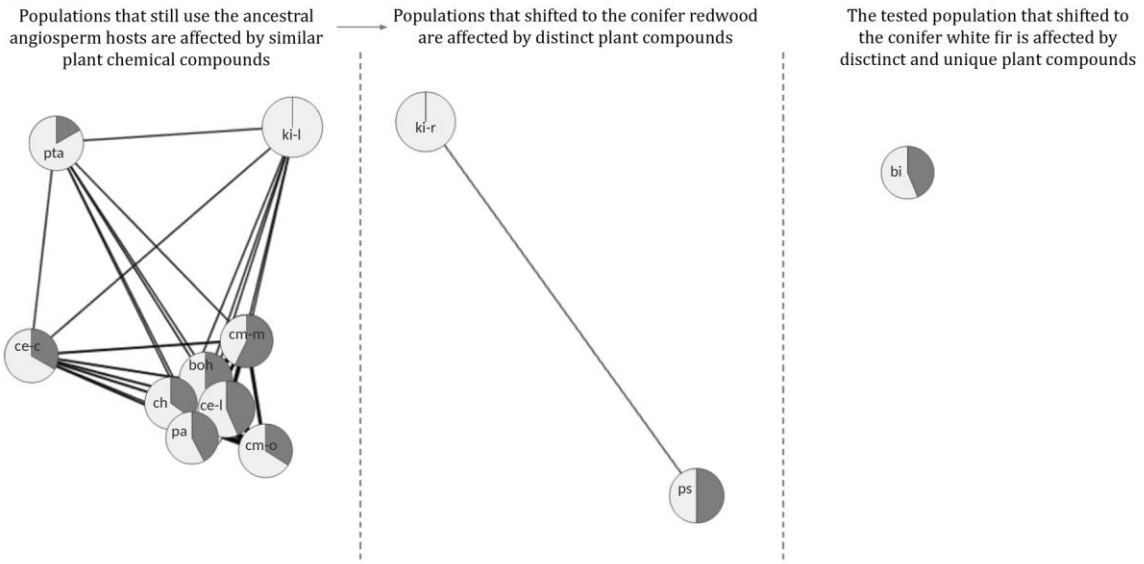


Figure 4. Similar chemical compounds affect performance of insects native to different angiosperm hosts, but different sets affect performances of insects native to conifers. Network built with Cytoscape 3.5.1 (Shannon *et al.* 2003). Circles in the network correspond to the twelve studied *Timema* populations. Individuals from populations that are close in the network are affected by similar sets of chemical compounds (the length of the edges connecting two populations is negatively proportional to the number of shared compounds affecting insect weight gain). The dashed lines separate groups of populations that are not affected by overlapping chemical compounds. *Timema* population name abbreviations are: bi: *T. bartmani* from white fir; boh: *T. boharti* from mahogany; cm-m: *T. californicum* from manzanita; cm-o: *T. californicum* from oak; ce-c: *T. cristinae* from chamise; ce-l: *T. cristinae* from lilac; ch: *T. chumash* from mahogany; ki-l: *T. knulli* from lilac; ki-r: *T. knulli* from redwood; pa: *T. podura* from chamise; ps: *T. poppensis* from redwood, pta: *T. petita* from lilac.

DISCUSSION

We analyzed the fundamental and realized feeding niches of *Timema* stick insects, which comprise a range of specialist to generalist species, in a phylogenetic framework. We showed that ecologically-specialized insects (occurring on a single or very few host species in natural populations) retained plasticity in host use and, surprisingly, featured broader fundamental feeding niches than ecological generalists. In line with classical theory, specialization at the fundamental niche level comes at a cost of reduced ability to use non-native host plants. However, this cost decreases with decreasing specialization, such that species with a generalist fundamental niche featured little or no trade-offs for adaptations to alternative host plants.

We identified two mutually non-exclusive mechanisms through which fundamental niche sizes expanded in *Timema*. First, the species that shifted to conifer hosts retained the ability to use plant groups used by their ancestors (i.e., angiosperms of the chaparral biome, especially lilac and chamise). Second, adaptation to particularly toxic hosts (i.e., redwood) allows insects to metabolize chemically diverse plants, including plants currently not used as hosts by any species of the *Timema* genus. In combination, these mechanisms can explain how generalist insect herbivores can evolve from specialists, a pattern detected repeatedly at the macroevolutionary scale (Schluter 2000; Janz *et al.* 2001, 2006; Nosil & Mooers 2005; Stireman 2005; Winkler & Mitter 2008). Furthermore, fundamental feeding niche expansions following host shifts should facilitate future host shifts in the same lineage, which could generate frequent host turnovers via positive feedback loops of host adaptation and range expansion. These processes are in accordance with the oscillation hypothesis (Janz & Nylin 2008) which suggests that insect

clades will feature successive phases of host range expansions and contractions. Thus generalist phenotypes could correspond to an evolutionarily transient phase, occasionally punctuating the tendency of lineages to increase specialization.

Our results suggest that the ability to use redwood is a key feeding innovation that allowed for range expansions in species that shifted to this host. Our feeding experiments showed that redwood is toxic to all *Timema* populations except for the native ones, while populations collected on redwood were able to survive and grow on all other tested host plants, without evidence for trade-offs. Only three *Timema* species are known to use redwood in nature: *T. poppensis* and *T. knulli* (used in the present study), and *T. douglasi*, an asexual species very closely related to *T. poppensis* (Table 1). According to the most recent *Timema* phylogeny (Riesch *et al.* 2017), the last common ancestor of these three species occurred approximately 6.6 million years ago, suggesting that the colonization of redwood happened around that time. The *Timema* genus appears to have originated in Southern California or Northern Mexico and expanded northward (Sandoval *et al.* 1998; Law & Crespi 2002b), with several range expansion events for the species currently occurring at the northern end of the distribution such as *T. poppensis* and *T. douglasi* (the exact distribution of *T. knulli* is not known). Therefore, the incorporation of redwood in their diet was very likely of paramount importance for these herbivores to be able to expand their range northward. Indeed, the geographic distribution of redwood spreads over 750 km along the Pacific coast of the United States (Farjon 2005), while reaching further north than most other *Timema* host plants.

While several ecological factors, such as competition, predation or limited dispersal (Futuyma & Moreno 1988; Agosta 2006; Agosta & Klemens 2008) can drive ecological

specialization, plant secondary chemistry has been brought forward as a key component driving insect performance and host plant specialization for herbivorous insects (Ehrlich & Raven 1964). In the present study however, adaptation to a particular host plant chemistry does not fully explain ecological specialization in *Timema*. Indeed, the performance of *Timema* individuals was typically not maximized on their native host plant, as previously shown in feeding experiments with chamise and lilac for insect populations adapted to these two plants (e.g., Sandoval & Nosil 2005; Nosil 2007). On the other hand, host plant chemistry might indirectly mediate host plant use by relaxing insect-insect competition. Indeed, redwood is an host for only few herbivore species, and is generally unaffected by regional outbreaks of herbivorous insects (Furniss 1977; Su & Tamashiro 1986; Grace & Yamamoto 1994). Furthermore, fires, being very common and an essential component of the Californian ecosystems (Minnich 1983; Brooks *et al.* 2004; Clinton *et al.* 2006), can favor redwood-insect association. Thanks to their thick bark, redwoods can easily withstand high levels of burning (Jacobs *et al.* 1985; Ramage *et al.* 2010). *Timema*, may thus survive fires while they would perish on more profitable hosts such as lilac or chamise. Thus, using redwood may be overall beneficial even if it represents a non-optimal food source.

Our analyses revealed only minor effects of phenolic and terpenic compounds on insect performance. Nonetheless, insect performances for populations native to angiosperms were significantly correlated to the phylogenetic distances between native and experimental host plants. Given that closely related plants share similar chemical defenses, chemistry determined by factors other than the simple additive effects of phenol and terpene compounds measured here is most likely a major driver of insect performance (Rasmann & Agrawal 2009; Richards *et al.* 2010).

In conclusion, our study provides new insights into the consequences of host shifts for the breadth of the fundamental feeding niche. These consequences are highly relevant as they influence the probability for additional host shifts and potential host-associated diversification. Specifically, we showed that the ability to use ancestral hosts is maintained following major host shifts (as when moving from angiosperms to conifers), and that adaptations to particularly challenging hosts is not necessarily associated with decreased performance on alternative hosts. To the contrary, we here showed that host shifts may broaden the breadth of the fundamental feeding niche. More generally, the joint analysis of fundamental and realized feeding niches in multiple related species provides unique insights into the evolutionary dynamics of host ranges in herbivorous insects.

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SUPPORTING INFORMATION

SI Methods

Plant chemical profile characterization

We extracted and quantified compounds in the phenolic and terpene classes of secondary metabolites from leaves of the seven plant species included in our experiments (i.e., lil, cha, oak, mz, df, wf, rdw; see Table 1), using methods adapted from (Pratt *et al.* 2014) and from (Moreira *et al.* 2015), for terpenes and phenolics, respectively. For each plant species, we extracted compounds from five independent replicates for both phenols and terpenes. Leave samples for terpene extractions were stored in the freezer (-20°C) prior to use, while samples for phenol extractions were dried in an oven at 45°C for one week. For phenol analyses, 100 mg of dried leaves per sample were reduced to powder with a pestle in liquid nitrogen, and phenols were extracted in 5 ml pure methanol (Sigma-Aldrich, CAS number 67-56-1). The methanolic solutions were kept at room temperature for 1 hour with continuous shaking. Thereafter, the extracts were sonicated for 10 minutes. Twenty-four hours later the tubes were centrifuged at 8000 rpm for 10 minutes and filtered. The collected supernatants were stored at 4°C until further use. Samples were analyzed by HPLC using a Grace C18 reversed phase column (3 µm, 150 × 4.6 mm; Grace Davison Discovery Science, Columbia, MD, USA) and an YL9100 instrument with diode array detection (YL Instrument Co., Anyang, Korea). The 15 µL injection was eluted at a constant flow of 0.7 mL min−1 with a gradient of acetonitrile and 0.25% phosphoric acid in water as follows: from 80% to 50% water in 5 min, then form 50% to 30% in 5 min, and kept at 30% for 7 min, and a final step from 30% to 5% in 4 min, followed by 5 min of equilibration time. Peaks were detected by a diode array detector at 270 nm (for hydrolizable tannins), 320 nm (for ferrulic acid derivates), 370 nm (for flavonoids) and 500 nm (for anthocyanins). Absorbance spectra were recorded from 200 to 900 nm. Peaks showing a characteristic absorption band of phenolics (Marbry *et al.* 1970) were recorded. Concentrations were calculated by using a standard curve that related peak areas to known gallic acid (for hydrolizable tannins), caffeic acid (for caffeic acid derivatives), quercitin (for flavonoids) and cyanidin (for anthocyanins) concentrations using 270 nm absorbance.

For terpene extractions, plant material was finely ground in liquid nitrogen and 250 mg were used for extraction in 2 mL n-hexane (Sigma-Aldrich, CAS number 110-54-3), with 20 ?l internal standard (IS) added (tetraline; Sigma-Aldrich, CAS number: 119-64-2, 198 ng in 10 ?l hexane). Five ?l of each sample were subsequently injected into a GC-MS (Agilent 6890 Gas Chromatograph coupled with a 5973N Mass Selective Detector; Agilent, Santa Clara, CA, USA) fitted with a 30 m 9 0.25 mm 9 0.25 lm film thickness HP-5MS fused silica column (Agilent). We operated the GC in splitless mode with helium as the carrier gas (flow rate 1 ml min-1). The GC oven temperature program was: 1 min hold at 50°C, 10°C min-1 ramp to 130°C, 5°C min-1 ramp to 180°C, 20°C min-1 ramp to 230°C and 1 min hold at 300°C. We identified terpenes using Kovats retention index from published work (Loayza *et al.* 1995) and by comparison with commercial standards when available. We measured the richness (total number of compounds) and total production of individual compounds as a proportion to the IS.

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S2 Complementary information for the Table. 1:

For plant genera without species specification, several species in the genus are used as hosts: *Abies* (*A. alba*, *A. concolor*), *Arctostaphylos* (*A. glauqua*, *A. obispoensis*, *A. patula*), *Ceanothus* (*C. cordulatus*, *C. cuneatus*, *C. crassifolius, C. divaricata*, *C. greggi* var. *perplexans*, *C. integerrimus*, *C. papillosus*, *C. sorediatus*, *C. spinosus*, *C. thyrsiflorus*), *Eriodictyon* (*E. californicum*, *E. trichocalyx* var. *lanetum*), *Juniperus* (*J. osteosperma*, *J. deppeana*, *J. californica*), *Pinus* (*P. attenuata*, *P. concorta*, *P. flexilis*, *P. monophylla*, *P. ponderosa*, *P. parryana*), *Quercus* (*Q. agrifolia*, *Q. chrysolepis*, *Q. dumosa*, *Q. durata*, *Q. wislinzenii*), *Rhamnus* (*R. californica*, *R. crocea*), and *Yucca* (*Y. schidigera*, *Y. whipplei*). The exact species of *Eriophyllum* is not known.

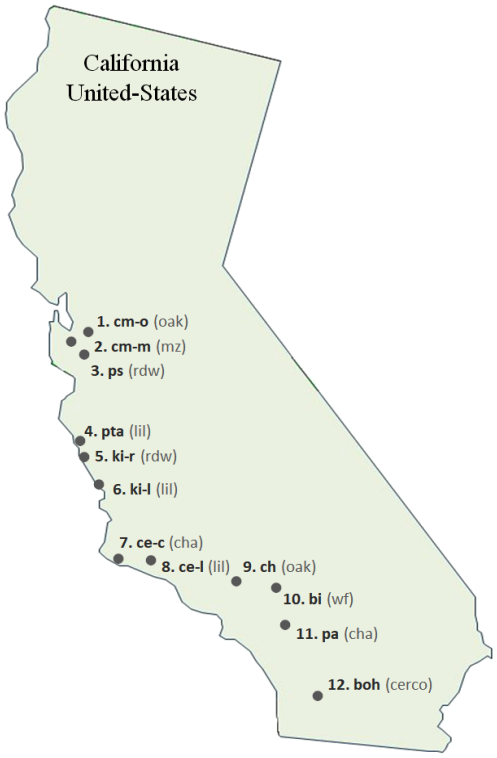
Table S1. Details about the populations of nine *Timema* species sampled. Number of individuals refer to the total number of individuals sampled in these locations on different host plants. For plant name abbreviations, see Table 1 in the main text.

Timema species	Location name (GPS coordinates)	Number of individuals per host plant sampled
T. bartmani	YMCA (34°09'48.8"N 116°54'22.6"W)	0 oak, 0 pin, 350 wf
T. boharti	Sunrise (32°58'40.6"N 116°31'27.7"W)	0 ad, 130 cer, 0 oak
T. californicum	Skyline (37°14'43.6"N 122°06'37.0"W)	2 ad, 18 mz, 43 oak
	Saratoga (37°11'47.0"N 122°02'27.1"W)	4 ad, 12 mz, 4 oak
	Summit (37°02'43.2"N 121°45'11.6"W)	51 mz, 0 oak, 0 rdw
T. chumash	HW2_1 (34°15'42.4"N 118°06'27.6"W)	45 cea, 70 cer, 250 oak
	HW2_2 (34°16'12.5"N 118°10'06.8"W)	18 ad, 5 cea, 11 oak
T. cristinae	Ojai1 (34°31'01.7"N 119°16'39.7"W)	245 cea, 73 cer, 6 mz, 70 oak, 5 toy
	Ojai2 (34°30'20.0"N 119°16'47.5"W)	23cea, 62 cer, 11 mz, 28 oak
	Ojai3 (34°31'59.6"N 119°14'51.8"W)	8 ad, 2 cea, 20 cer, 8 oak
	WTA1 (34°30'46.6"N 119°46'41.7"W)	597 ad, 317 cer, 78 oak
	WTA2 (34°30'22.3"N 119°46'05.3"W)	81 ad, 1 cer, 8 mz, 9 oak, 2 toy
	WTA3 (34°30'56.8"N 119°46'43.7"W)	60 ad, 24 cea, 5 mz, 7 toy
T. knulli	HW1_1 (36°10'6.899''N 121°40'56.64''W)	0 ad, 9 cea, 0 oak, 0 rdw
	HW1_2 (36°14'50.8"N 121°46'54.4"W)	0 cea, 0 oak,13rdw
	Big Creek (36°4'15.661''N 121°32'44.041''W)	12 cea, 0 mz, 0 oak, 0 rdw
T. petita	HW1_3 (36°29'10.0"N 121°55'56.2"W)	330 cea, 3 mz, 0 oak
T. podura	Indian (33°47'50.5"N 116°46'35.5"W)	79 ad, 60 cea, 0 cer, 7 mz, 0 oak
	Poppet (33°51'36.9"N 116°50'20.4"W)	45 ad, 0 cea, 0 mz
T. poppensis	Fish_Rock (38°49'05.1"N 123°35'03.5"W)	0 cea, 137 df, 14 rdw
	Bear Creek (37°09'56.2"N 122°00'56.4"W)	85 df, 0 oak, 35 rdw
	Madonna (37°01'07.5"N 121°43'32.0"W)	0 mz, 0 oak, 403 rdw

Table S2. Relative survival of *Timema* individuals on different plants during ten days. For each *Timema* population, the survival on the native host plant is highlighted in grey. In the case of *T. boharti* and *T. chumash* the survival on their native host plant (*Cercocarpus betuloides*) is unknown as this plant was not included in the experiments. The proportion of deviance accounted for by the different plants in the GLMs was calculated using the modEva R package (Barbosa *et al.* 2013); Pearson's chi-squared tests were performed to test whether plants explain a significant amount of deviance (p-value < 0.001: *** ; < 0.01: ** ; < 0.05: *). For plant name abbreviations, see Table 1 in the main text.

Timema species	**Sample size per treatment**	**lil**	**cha**	**oak**	**mz**	**df**	**wf**	**rdw**	**% of deviance explained**	**p-value**
T. bartmani	14 to 80	100.0	52.5	0.0	37.7	37,7	35.4	0.0	18.0	6.0e-07***
T. boharti	10	100.0	88.9	55.6	33.3	22.2	0.0	0.0	43.7	9.3e-06***
T. californicum-mz	10	90.0	100.0	100.0	90.0	70.0	50.0	10.0	42.1	2.4e-06***
T. californicum-oak	10 to 20	100.0	100.0	100.0	100.0	100.0	80.0	50.0	47.5	4.3e-04***
T. chumash	10	88.9	100.0	88.9	100.0	55.6	77.8	55.6	10.9	0.18
T. cristinae-cha	10	100.0	87.5	25.0	75.0	75.0	12.5	0.0	44.7	2.3e-05***
T. cristinae-lil	10	100.0	100.0	57.9	84.2	34.7	28.9	11.6	31.3	3.3e-04***
T. knulli-lil	10 to 20	100.0	100.0	26.7	93.3	26.7	26.7	6.7	29.7	3.9e-07***
T. knulli-rdw	24	90.5	86.7	71.4	77.5	100.0	82.1	86.7	3.9	0.36
T. petita	10	100.0	90.0	20.0	90.0	30.0	30.0	10.0	34.1	4.7e-05***
T. podura	15	76.9	100.0	23.1	92.3	23.1	7.7	0.0	41.1	4.7e-09***
T. poppensis	30	92.9	85.7	60.7	78.6	100	64.3	75.0	7.6	0.009**

Barbosa, A.M., Brown, J.A. & Real, R. (2013). ModEvA–an R package for model evaluation and analysis.



GPS coordinates

1. 37°20'41.3''N 121°37'59.6''W
2. 37°13'20.6''N 122°05'16.3''W
3. 37°00'40.2''N 121°42'48.5''W
4. 36°29'10.0''N 121°55'56.2''W
5. 35°50'10.3''N 121°23'29.3''W
6. 35°46'27.3''N 121°19'34.1''W
7. 34°30'53.2''N 119°46'45.3''W
8. 34°30'19.7''N 119°16'53.6'W
9. 34°16'13.0''N 118°10'07.4''W
10. 34°09'48.8''N 116°54'22.6''W
11. 33°41'12.3''N 116°42'11.2''W
12. 32°58'40.6''N 116°31'27.7''W

Figure S1. Geographical locations of the *Timema* populations used. Population labels are written in bold and with the host plant indicated in brackets. Population name abbreviations are: bi: *T. bartmani* from white fir; boh: *T. boharti* from montain mahogany; cm-m: *T. californicum* from manzanita; cm-o: *T. californicum* from oak; ce-c: *T. cristinae* from chamise; ce-l: *T. cristinae* from lilac; ch: *T. chumash* from oak; ki-l: *T. knulli* from lilac; ki-r: *T. knulli* from redwood; pa: *T. podura* from chamise; ps*: T. poppensis* from redwood, pa*: T. petita* from lilac.

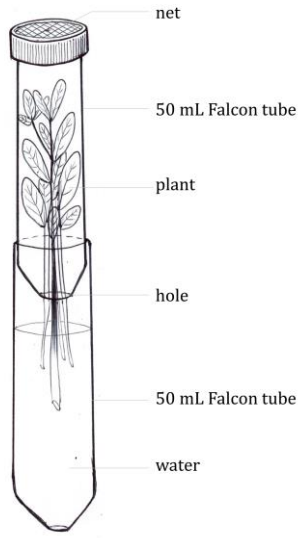


Figure S2. Illustration of the experimental system used to perform the feeding experiment. To measure the performance of insects on different plants, the collected juveniles were transferred to 50mL Falcon tubes containing a branch, with the broken end immersed in a water reservoir. Prior to the transfer, individual insects were weighed with an analytical balance (Kern ABT 120-5DM). During the ten days of the experiment, all tubes were observed daily to verify the survival of individuals and individuals that survived were weighted again at the end of the experiment.

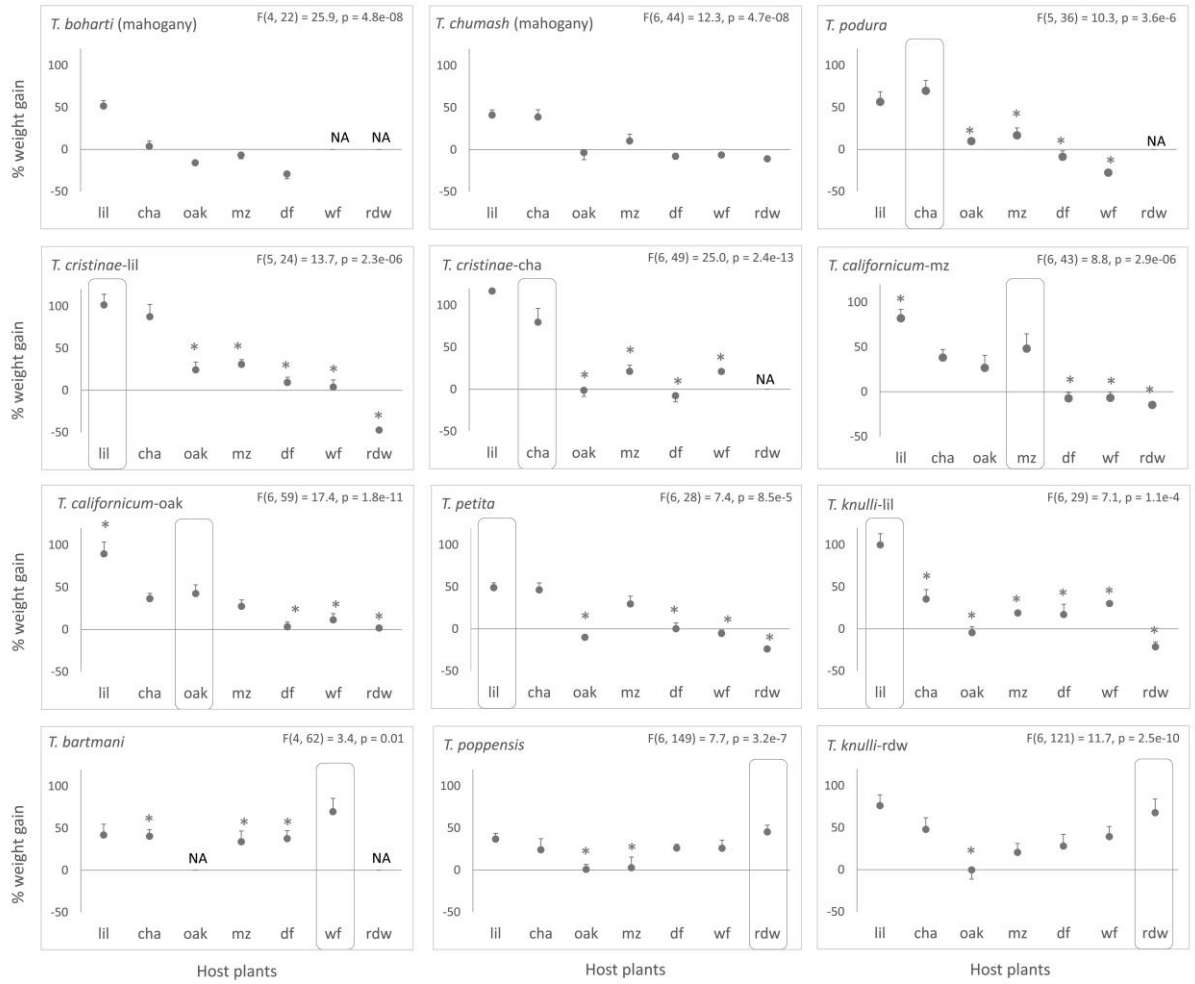


Figure S3. Percentages of weight gain for individuals fed with different plants for ten days. Each panel corresponds to a different *Timema* population, native hosts are enclosed in rectangles. For each population, the amount of weight gained by individuals that survived during ten days on the different plants was compared using one-way ANOVAs. The asterisks indicate the plants on which the performance is significantly different from their performance on the native host (planned comparisons; * significant at $p<0.05$). For some plant by *Timema* population combinations, there are no weight gain data (NA) because all individuals died before the end of the experiment. For plant name abbreviations, see Table 1 in the main text.

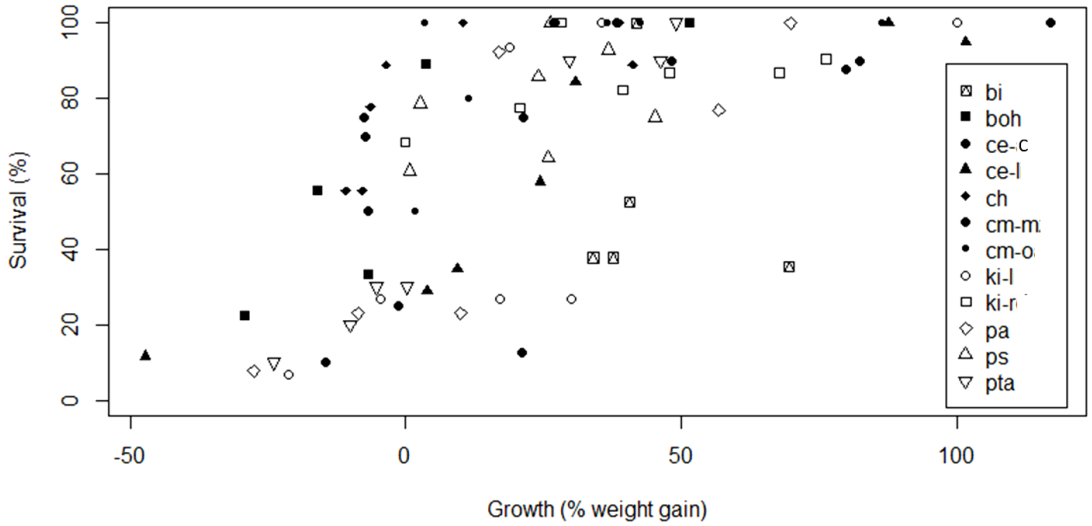


Figure S4 Correlation between survival and weight gain of individuals from the twelve *Timema* populations. Each symbol corresponds to a different *Timema* population (weight gain averaged across all individuals). For *Timema* population labels, see Fig. S1.

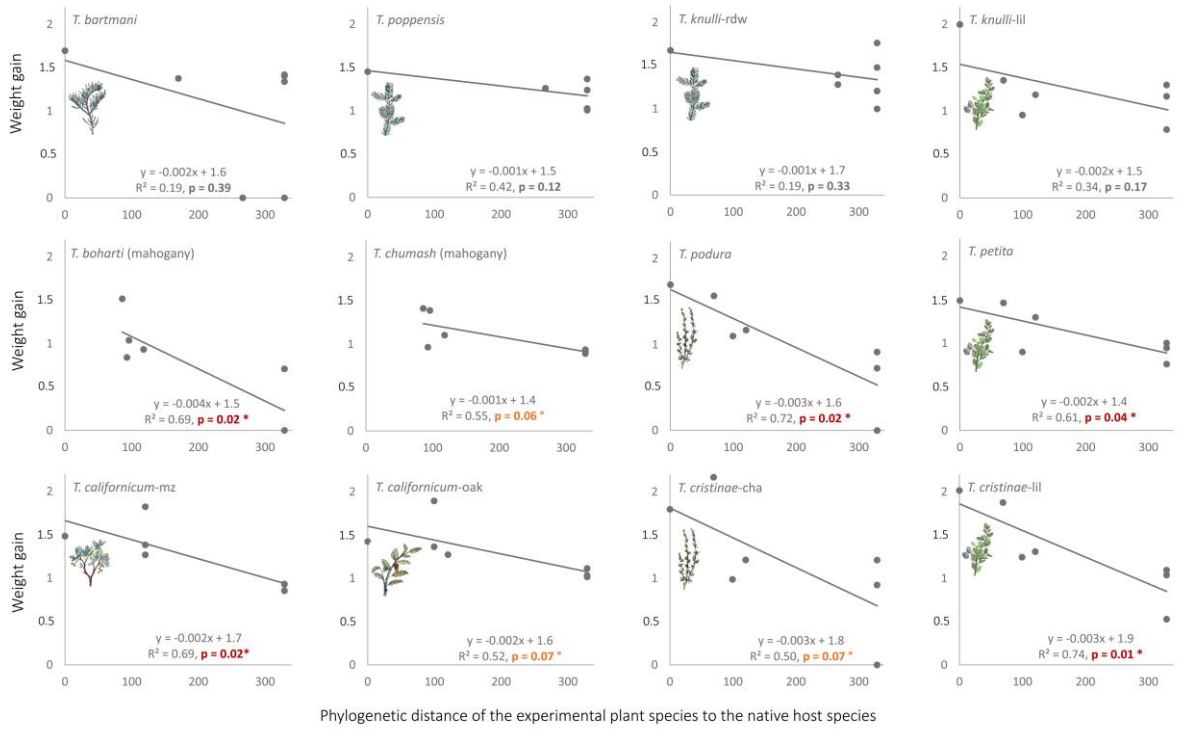


Figure S5. Relative weight gain of *Timema* individuals as a function of the phylogenetic distance between the native host plant and the plants used in the experiments. Native plants are indicated with icons (except for *T. boharti* and *T. chumash*). See Table 1 in the main text for phylogenetic distances among plants. Steeper slopes indicate more extensive trade-offs.

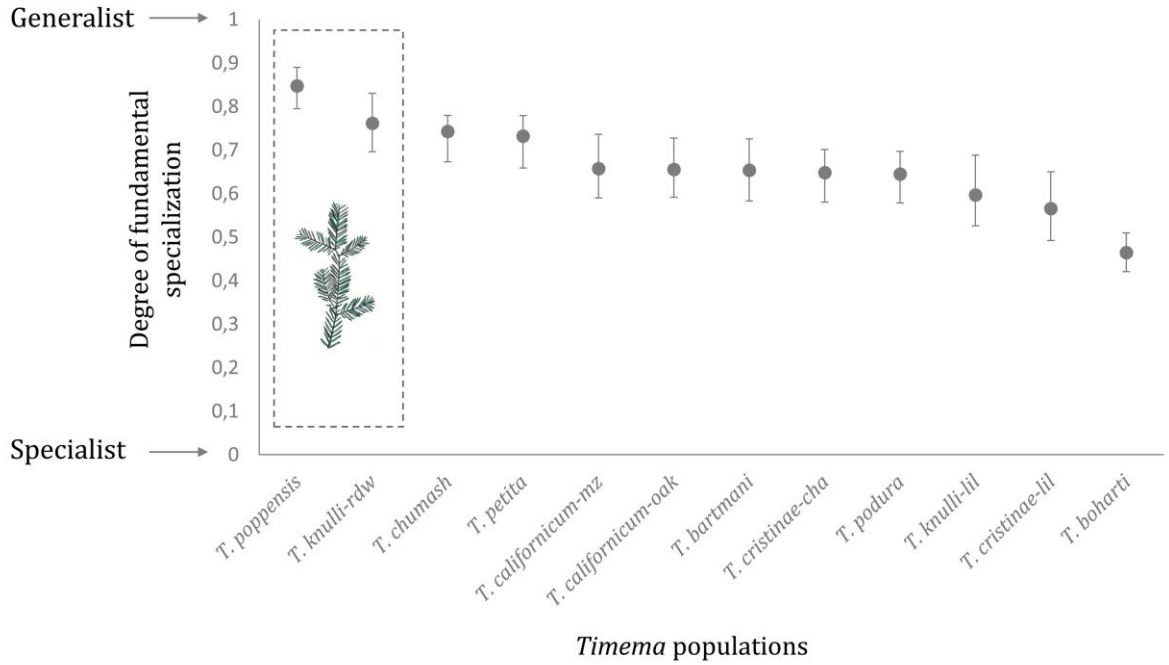


Figure S6. Breadth of the fundamental feeding niche of insect herbivores. Niche breadth is quantified via the specificity index Tau (with 95% CI), based on insect weight gain on different plants (data from redwood excluded). The insect populations are listed from the least to the most specialist; *T. poppensis* and *T. knulli* native to redwood remain the most generalist populations even if data from redwood are excluded.

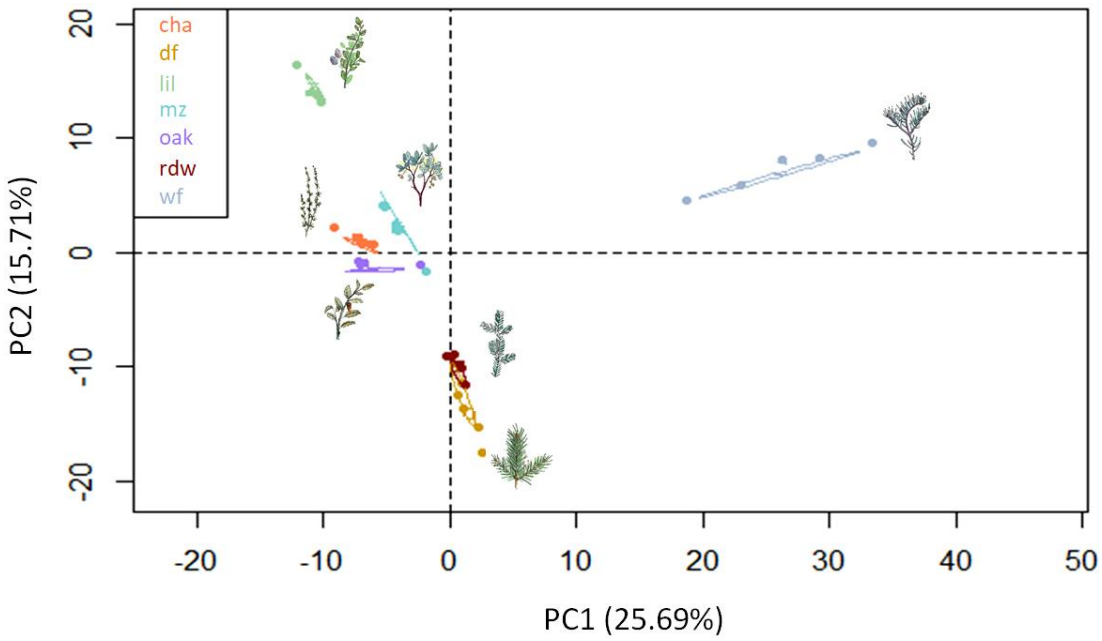


Figure S7. Principal component analysis based on the 521 plant chemical compounds (28 phenolic and 493 terpenic compounds). Percentages indicate the amount of variance explained by each axis. For plant name abbreviations, see Table 1 in the main text.

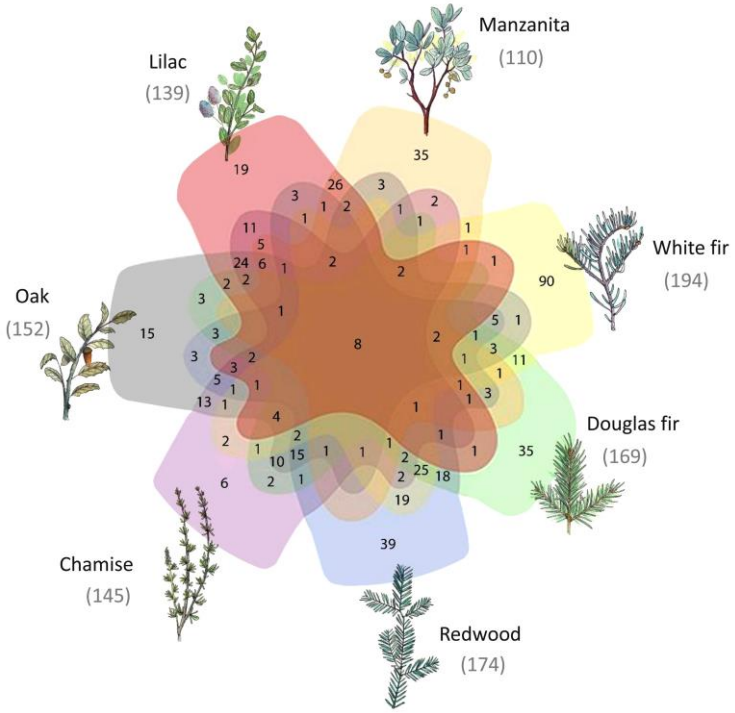
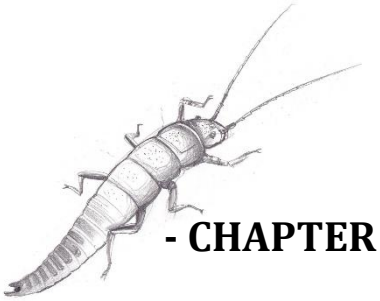


Figure S8. Specific and shared chemical compounds of different *Timema* host plants. The numbers in the Venn diagram indicate the number of terpenic and phenolic compounds shared among sets of plants and the number of species specific ones. Numbers in brackets indicate the total number of chemical compounds present in each plant.



- CHAPTER II -

Fundamental and realized niche breadths in sexual and asexual stick-insects

Running head:
Niches evolution in asexual insects

C. Larose, D. J. Parker and T. Schwander

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Author Contributions: CL and TJ designed the study. CL and DP conducted fieldwork and performed the experiments. CL analysed the data. CL and TS wrote the manuscript, with input from all co-authors.

ABSTRACT

Understanding the factors that contribute to the maintenance of sex and that limit the success of other reproductive modes is a major challenge in evolutionary biology. Ecological divergences between lineages with different reproductive modes could help to maintain reproductive polymorphism at least transiently but there is little empirical information on the consequences of asexuality for the evolution of ecological niches. In this study, we investigate how niche breadths evolve following transitions from sexual reproduction to asexuality. Specifically, we estimated and compared the realized feeding niche breadth of five independently derived, asexual *Timema* stick insect species and their sexual relatives. We found that asexual species had a systematically narrower realized niche than sexual species. To develop insights into how the narrower realized niches of asexual versus sexual species come about, we quantified the breadth of their fundamental niches but found no systematic differences between reproductive modes. The narrow realized niches found in asexuals are thus likely a consequence of biotic interactions that constrain realized niche size in asexuals more strongly than in sexuals. Interestingly, the fundamental niche was significantly broader in the oldest asexual species compared to its sexual relative. Relatively broad ecological tolerances may help explain how this species has persisted over more than a million years in the absence of sex.

Keywords: *Timema* stick insects, Sexual versus asexual reproduction, Specialization, Realized versus fundamental niche.

INTRODUCTION

The maintenance of obligate sex in natural populations, despite numerous disadvantages compared to other reproductive systems, is a major evolutionary paradox. Although there is a rich body of theory proposing potential benefits of sex (e.g., Muller 1964; Hill & Robertson 1966; Hamilton 1980; Kondrashov 1988; Barton & Charlesworth 1998; Otto & Lenormand 2002), empirical data evaluating potential benefits under natural conditions remain scarce (reviewed in Neiman *et al.* 2018). In particular, there is a paucity of studies that examine the situations and mechanisms that allow for the maintenance of reproductive polymorphisms between obligate sexual and asexual species. A simple mechanism that could facilitate the maintenance of reproductive polymorphisms is niche differentiation between sexual and asexual species (Meirmans *et al.* 2012). Such niche differentiation could result from a difference in ecological optima between sexuals and asexuals (e.g., Case & Taper 1986), or situations where sexual species cover larger fractions of the available niche space than their asexual counterparts (e.g., Bell 1982).

A species may occupy a wide range of environments either because individuals are generalists through phenotypic plasticity in habitat use, or because a species comprises different genotypes, each specialized in its habitat use (Van Valen & Grant, 1970). Because asexual species derive from sexual ancestors, fundamental niches (i.e., the range of conditions allowing for survival, growth and reproduction) in new asexual species should depend directly on the fundamental niche found in the ancestral sexual species. How the distribution of fundamental niches in an ancestral sexual population translates to that found in an asexual population is however unclear. For example, the *Frozen Niche Variation* model (FNV) predicts that the phenotypic distribution of a new, recently

derived asexual would be narrower than that of its genetically variable sexual ancestor, because a single sexual genotype will be “frozen” in the new asexual clone (Vrijenhoek 1984; Case & Taper 1986; Case 1990; Weeks 1993). This may result in different fundamental niche breadths with the new asexual clone being able to exploit fewer niches, and thus being more specialized, than the sexual species as a whole (Vrijenhoek 1984; Vrijenhoek & Parker Jr 2009). By contrast, the "*General-Purpose Genotype*" hypothesis (GPG; Lynch 1984 but see also White 1973; Parker et al., 1977) proposes that asexual clones should generally have broader environmental tolerances than sexual individuals because of strong selection for phenotypic plasticity in asexuals. Indeed, a temporally and spatially variable environment should favor, among all the independently derived asexual clones, those with broad environmental tolerances. Under this scenario, we would expect asexual populations to have broader ecological niches than sexual ones (Fig. 2). The two hypotheses are non-mutually exclusive. For example, combining the FNV and GPG, we can suggest that young, independently derived clones feature, on average, narrow niches, while old clones would feature broad niches.

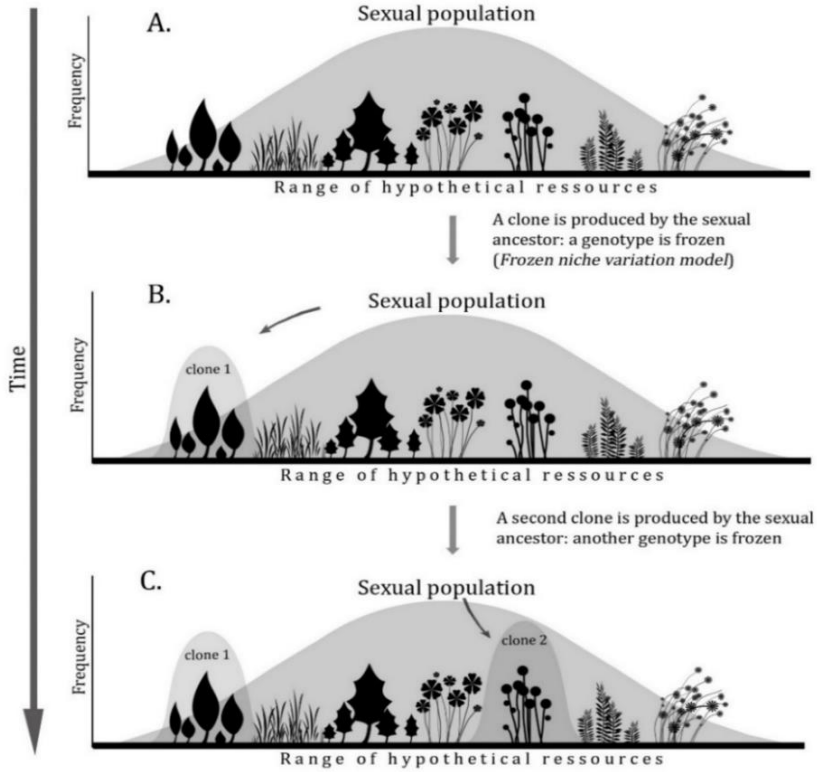

Figure 1. The frozen niche variation model. (A) A sexual population (broad curve) exhibits genetic variation for the use of a natural resource (here symbolized by a range of hypothetical plants). (B) A new asexual clone is produced, comprising a small subset of the genotypic diversity contained in its sexual ancestor (C) A second clone is produced from a different sexual genotype characterized by a different ecological niche. The niche breadth of the sexual population as a whole is larger than the one of each individual clone. Figure modified from Vrijenhoek and Parker, 2009.

Regarding the breadth of the realized niche (i.e., the part of the fundamental niche used by organisms under natural conditions), there is currently no specific theory predicting similarities or differences between sexuals and asexuals. There are however several theories predicting that sex may accelerate the rate of adaptation compared to asexuality (Hill & Robertson 1966; Kondrashov 1988; Barton & Charlesworth 1998; Otto & Lenormand 2002). Sexual organisms therefore may be better able to evolve counter-adaptations to competitors, pathogens, or predators than asexuals. As a consequence, the realized niche in asexual organisms may be much smaller than in sexual organisms due to reduced ability to respond to these biotic pressures.

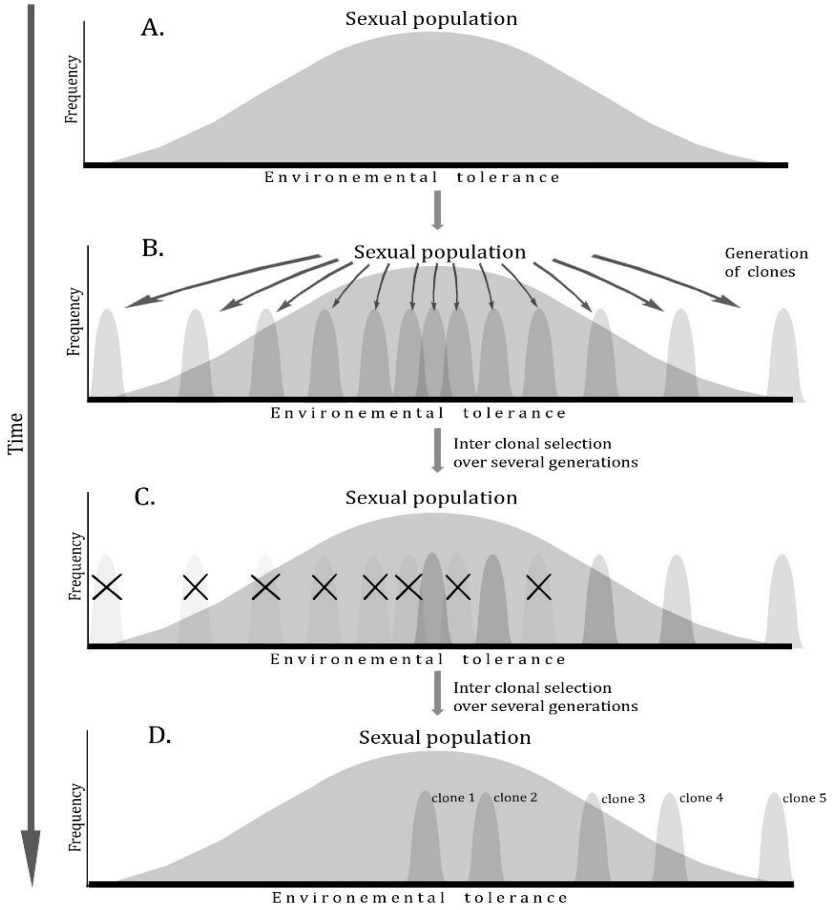

Figure 2. General purpose genotype hypothesis. (A) Individuals in a sexual population vary in the range of their environmental tolerances (narrow to broad phenotypic plasticity) (B) Clones are produced from different genotypes in the sexual population with different levels of phenotypic plasticity. (C and D) Natural selection favors clones with broader tolerances such that clones may feature higher levels of phenotypic plasticity than the sexual population as a whole (e.g. extreme case of clone 5). Figure adapted from Niklassom,1995 and Vrijenhoek and Parker, 2009.

Here we evaluate whether asexuality is associated with different niche characteristics than sexual reproduction using herbivorous stick insects of the genus *Timema* as a model system. Seven independently derived asexual lineages have been identified in this genus, each with a closely related sexual counterpart (Law & Crespi 2002a; Schwander *et al.* 2011). This allows us to perform replicate comparisons between sexual and asexual lineages. Moreover, the asexual *Timema* lineages vary in age (Law & Crespi 2002a; Schwander *et al.* 2011; Bast *et al.* 2018), allowing us to assess possible consequences of asexuality over a range from recently derived to long-term asexuality.

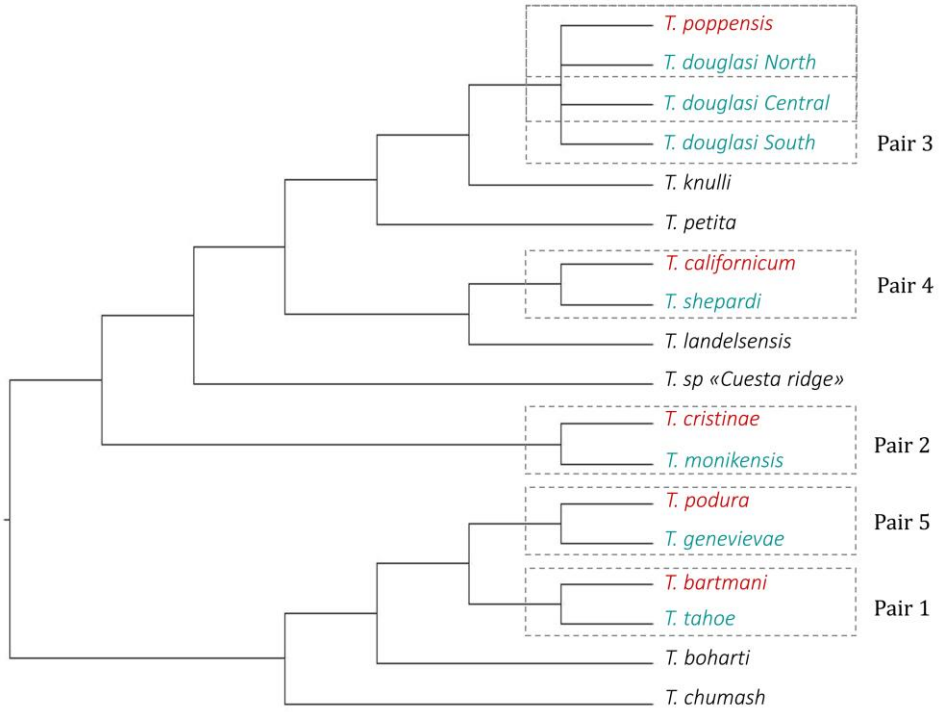

Figure 3. *Timema* phylogeny highlighting the sexual and asexual species. Phylogeny redrawn from Riesch *et al.* (2017) with the seven asexual lineages added from Schwander *et al.* 2011 (in blue). The used sexual species are labeled in red. Pair numbers correspond to the most recent (Pair 1) to the most ancient (Pair 5) transition to asexuality (ranking from Bast et al. 2018).

Timema species feed on the leaves of very diverse host plants, comprising both angiosperms and conifers (Larose et al *in review*), and different cryptic morphs have evolved on different host plants, both within and between species (Sandoval 1994a, b; Nosil 2007; Sandoval & Crespi 2008). Intra-specific color polymorphism in *Timema* has a genetic basis, at least for the subset of morphs for which it has been investigated (Sandoval 1993; Comeault *et al.* 2015). Moreover, previous studies in *Timema* have shown that the combination of selection imposed by predators and *Timema* host preference maintain a correlation between morph frequency and host-plant frequency between populations (Sandoval 1994a; Nosil 2004; Sandoval & Nosil 2005).

We investigated if sexual and asexual species and populations differ in terms of realized and fundamental niches and niche breadths. We first estimated the size of the realized feeding niches as well as color polymorphism of sexuals and asexuals both at the species and at the population level in five sexual-asexual *Timema* sister species pairs, using occurrences on different host plants in natural populations. We then conducted feeding experiments with species from four sexual-asexual species pairs to characterize fundamental feeding niches. For these experiments, we reared individuals from each species on seven different host plants and assessed their respective performances on different plants. In combination, these experiments allowed us to evaluate the consequences of different reproductive modes on fundamental and realized niches, and help us understand the role that niche breadth may have on the overwhelming success of sex.

METHODS

Realized feeding niche breadths

Data from a previous study that collected information on host plant use across all 23 known *Timema* species (Larose et al. *in review*) allowed us to estimate the size of the realized feeding niche of sexuals and asexuals at the species level. To estimate the realized niche at the population level, we further performed a count of the number of individuals collected on each potential host plant used in the field across 30 populations from five species pairs (between two and six populations per species; Table. S1). We only selected localities in which at least three plants from the 25 known *Timema* host plants (Larose et al. *in review*) were present.

To quantify the size of the realized feeding niche at the population level, we used the inversed Tau (τ) specialization index (Yanai *et al.* 2004), which ranges from 0 (pure specialist) to 1 (complete generalist).

Degree of color polymorphism

In order to develop insights into the factors generating variation in realized feeding niche breadths, we investigated the relationship between color polymorphism and realized niche at the species and population level. Color phenotypes vary broadly in several *Timema* species but can be separated into a total of 14 discrete morphs across all species (range 1-8 per species; Table S1). We recorded color morph frequencies from all sampling locations (Table. S2) and used the Simpson diversity index to quantify the level of polymorphism (Simpson 1949). This index varies between 0 (here indicating color monomorphism) and 1 (indicating diversity of equally frequent color morphs). We then

estimated the correlation between the degree of color polymorphism and the size of the realized feeding niche, both at the species and at the population levels. We used Phylogenetic generalized least squares (PGLS) analyses to account for phylogenetic non-independence among *Timema* species. These analyses were conducted using the ape (Paradis *et al.* 2004) and nlme (Pinheiro *et al.* 2009) R packages (R Core Team 2017) using a Brownian motion model for trait evolution.

Fundamental feeding niche breadths

To estimate the fundamental feeding niche breadth of sexual and asexual *Timema* species, we performed a feeding experiment and measured insect performance on different host plants. We chose seven plants known to be commonly used by several *Timema* species, while trying to cover the phylogenetic diversity of the host plants (Larose et al. *in review*): specifically we chose four angiosperms (*Ceanothus thyrsiflorus* (lilac, lil); *Adenostoma fasciculatum* (chamise, cha); *Quercus agrifolia* (oak); and *Arctostaphylos glauca* (manzanita, mz)), and three conifers (*Pseudotsuga menziesii* (douglas fir, df); *Abies concolor* (white fir, wf), and *Sequoia sempervirens* (redwood, rdw)). Stick insects from eight *Timema* species from four sexual-asexual species pairs were collected throughout California (Table 1). We only used fourth-instar juvenile females for feeding experiments because the younger stages have a much lower survival rate, especially under the stress of experimental manipulation and to minimize age-related effects on insect performance during our experiments. Between 10 and 20 such females were used per host plant to measure survival and weight gain during 10 days, for a total of 70-105 females per population (635 insects in total; Table 1).

Table 1. Overview of the *Timema* sp used in this study

Timema species	Reproductive mode	Original host plant	GPS coordinates	Number of individuals[1]
T. cristinae	Sexual	*Ceanothus thyrsiflorus*	34°30'19.7''N 119°16'53.6''W	70
T. monikensis	Asexual	*Cercocarpus betuloides*	34°06'53.7''N 118°51'09.7''W	100
T. poppensis	Sexual	*Pseudotsuga menziesii*	37°09'56.7''N 122°00'55.0''W	70
T. douglasi	Asexual	*Pseudotsuga menziesii*	38°58'57.2''N 123°28'10.4''W	70
T. californicum	Sexual	*Arctostaphyllos glauca*	37°20'41.3''N 121°37'59.6''W	80
T. shepardi	Asexual	*Arctostaphyllos glauca*	39°12'02.8''N 123°17'38.2''W	70
T. podura	Sexual	*Adenostoma fasciculatum*	33°41'12.3''N 116°42'11.2''W	105
T. genevievae	Asexual	*Adenostoma fasciculatum*	37°19'42.0''N 121°29'07.6''W	70

[1] number of individuals used in the feeding experiment

We first used a generalized linear model (GLM) with a binomial error to compare survival and an ANOVA to compare the weight gain of all stick insects species on the different plants using R (R Core Team 2017). We then compared for each *Timema* species pair separately, the survival and weight gain of the sexual and asexual individuals, testing specificaly for an interaction between reproductive mode and plant species, because a significant interaction between these two factors would indicate different fundamental niches between sexuals and asexuals.

We finally quantified the breadth of the fundamental feeding niche of eight species using again the inversed Tau specialization index (Yanai *et al.* 2004), where **n** corresponds to the number of *Timema* host plants tested (i.e, seven plants), **xi** represents the weight gain on plant i, and **max (xi)** is the highest weight gain for the focal population. While this index is robust even for very small sample sizes (Kryuchkova-Mostacci & Robinson-rechavi 2016), it needs positive values to be calculated. We therefore transformed our percentages of weight gain (which are negative when individuals lose weight), to relative weights of insects at the end of the feeding trials (i.e., an insect that lost 20% of its weight during the trial would be assigned the value 0.8, while one that gained 20% would be

assigned 1.2). We could not compare the fundamental niche of the *T. bartmani*/*T. tahoe* species pair because *T. tahoe* individuals of the appropriate developmental stage could not be collected in sufficient numbers for the feeding experiment.

RESULTS

Realized feeding niche breadths

In order to compare the realized feeding niche breadth (i.e., the host plants used under natural conditions) of the sexual and asexual species, we performed a basic count of the plants on which they have been observed in the wild (data from Larose et al., *in review*). A fairly obvious pattern emerged, as we found that in four out of five cases the sexuals used at least twice as many plants as their asexual relatives (Fig. 4) and are therefore more ecologically generalist. In the remaining case (*T. poppensis/T. douglasi*), the sexual and the asexual species used the same number of host plants in the wild (Fig. 4).

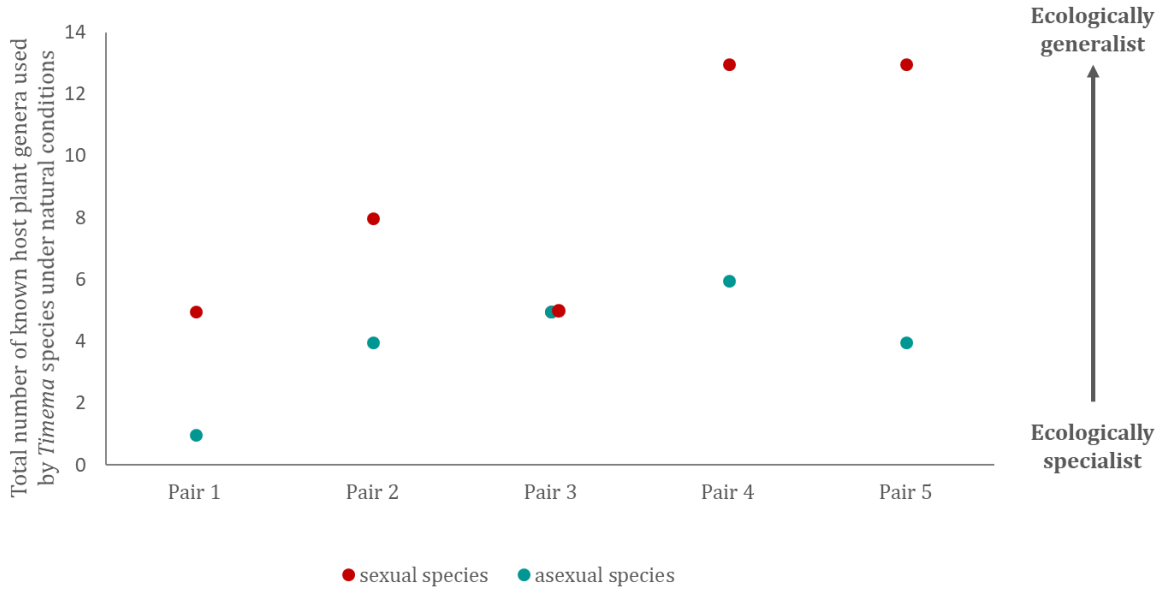

Figure 4. Realized feeding niche breadths of sexual and asexual *Timema* stick insects, measured at the species level. The pairs are listed from the most recent to the most ancient transition to asexuality (ranking from Bast *et al.* 2018).

In addition, we estimated the realized feeding niche breadths of ten *Timema* species at the population level (Table S1). We found that all ten species are somewhat specialist at the population level (Tau indices varying between 0.52 and 1 ; Fig. 5B). Overall, we did not

find significant differences in the degree of specialization between sexual and asexual populations (GLM; p-value = 0.19). However, sexual populations vary more than asexual ones in their degree of specialization (Levene's test, $F_{1, 27} = 12.2$, p-value < 0.002; Fig. 5B).

Given the extensive variation in terms of realized feeding niche of different *Timema* species and populations, we aimed to develop insights into the factors generating this variation. We thus compared color polymorphism within *Timema* species and populations sampled in this study with their degree of ecological specialization. At the species level, the realized niche was correlated with the number of morphs present within these species (correlation corrected with Phylogenetic Generalized Least Squares (PGLS); r= 0.57, p-value < 0.003; Fig. S1). Similar to the size of the species-level realized niche, the asexuals contain two to five times fewer morphs than their sexual relatives, with the exception of *T. poppensis/T. douglasi*, in which both species have the same degree of color polymorphism. By contrast, at the population level, we did not detect any link between color polymorphism and the size of the realized feeding niche (Pearson's correlation; r = 0.14, p-value = 0.46; Fig. S1B).

Fundamental feeding niche breadths

In order to investigate if the decrease in the degree of ecological specialization observed in asexual species is due to a reduction in their fundamental feeding niche breadth, we performed a feeding experiment. First, this experiment showed that survival and weight gain vary widely among the different studied *Timema* species when fed with different plants (p < 2.2e-16 for survival and $F_{7, 292} = 8.94$, p < 5.5e-10 for weight gain; Fig. 6). In each species pair separately, we then modeled the survival and weight gain as functions of the species' reproductive mode and of the experimental feeding treatments (with

interaction term). A significant interaction in a given species pair would indicate that sexual and asexual species have different fundamental feeding niches. We found a significant interaction for the pair *T. californicum* - *T. shepardi*, however this was only the case for survival, and only a trend for weight gain. We also found a significant interaction for the pair *T. poppensis* - *T. douglasi*, but only for weight gain, not survival (Table. 2). In addition, we found a marginally non-significant interaction for weight gain in the species pair *T. podura* - *T. genevievae* (Table. 2). These results suggest that in two or three species pairs, asexuals and sexuals may have diverged in their fundamental niches.

Table 2. Effect of experimental feeding treatments and reproductive mode on survival and weight gain.

***Timema* species pair**	**Factors tested in the statistical models**	**Survival**	**Weight gain**
Pair2:	[Reproductive mode]	1.06×10^{-05} ***	$F_{(1, 34)} = 3.9$, p = 0.054 •
T. cristinae /	[Feeding treatment]	2.856×10^{-09} ***	$F_{(5, 34)} = 14.8$, $p = 9.97 \times 10^{-08}$ ***
T. monikensis	[Reproductive mode: Feeding treatment] interaction	0.59	$F_{(2, 34)} = 3.9$, p = 0.222
Pair3:	[Reproductive mode]	0.33	$F_{(1, 107)} = 4.9$, p = 0.03 *
T. poppensis /	[Feeding treatment]	0.20	$F_{(6, 107)} = 13.1$, $p = 4.6 \times 10^{-11}$ ***
T. douglasi	[Reproductive mode: Feeding treatment] interaction	0.44	$F_{(6, 107)} = 5.5$, $p = 5.4 \times 10^{-05}$ ***
Pair4:	[Reproductive mode]	0.009 ***	$F_{(1, 71)} = 13.7$, p = 0.0004 ***
T. californicum	[Feeding treatment]	4.8×10^{-05} ***	$F_{(6, 71)} = 19.4$, $p = 2.9 \times 10^{-13}$ ***
/ T. shepardi	[Reproductive mode: Feeding treatment] interaction	0.0009 ***	$F_{(6, 71)} = 1.9$, p = 0.09 •
Pair5:	[Reproductive mode]	0.0004 ***	$F_{(1, 80)} = 4.4$, p = 0.04 *
T. podura /	[Feeding treatment]	6.4×10^{-19} ***	$F_{(6, 80)} = 22.1$, $p = 3.5 \times 10^{-15}$ ***
T. genevievae	[Reproductive mode: Feeding treatment] interaction	0.35	$F_{(5, 80)} = 2.1$, p = 0.08 •

p-value < 0.001: *** ; < 0.01: ** ; < 0.05: *; <0.1: •

To quantify the breadth of the fundamental feeding niche of each *Timema* species, we calculated Tau indices from survival and weight gain of individuals fed with the different plants during ten days. These two indices were strongly correlated (Pearson's correlationt=, r = 0.96, p < 0.0001; Fig. 6). We found significant differences in the fundamental niche breadths of sexuals compared to asexual species in two species pairs, (*T. cristinae*/*T. monikensis* and *T. podura*/*T. genevievae*; Fig. 5, Fig. 6) while the remaining two pairs (*T. poppensis*/*T. douglasi* and *T. californicum*/*T. shepardi*) showed no significant

difference (Fig. 6). Interestingly, *T. monikensis* and *T. genevievae*, which represent the most recent asexual lineage and oldest asexual lineage tested respectively, were characterized by an opposite result. *T. monikensis* was significantly more specialist (Tau based on weight gain = 0.73, 95% CI 0.71 - 0.78 and Tau based on survival = 0.79, 95% CI 0.71 - 0.87) than it sexual relative *T. cristinae* (Tau based on weight gain = 0.53, 95% CI 0.45 - 0.59 and Tau based on survival = 0.54, 95% CI 0.42 - 0.66; Fig. 6). On the contrary, the ancient asexual *T. genevievae* was significantly more generalist (Tau based on weight gain = 0.23, 95% CI 0.18 - 0.29 and Tau based on survival = 0.22, 95% CI 0.12 - 0.32) than it sexual sister species *T. podura* (Tau based on weight gain = 0.46, 95% CI 0.42 - 0.52 and Tau based on survival = 0.63, 95% CI 0.53 - 0.73; Fig. 6).

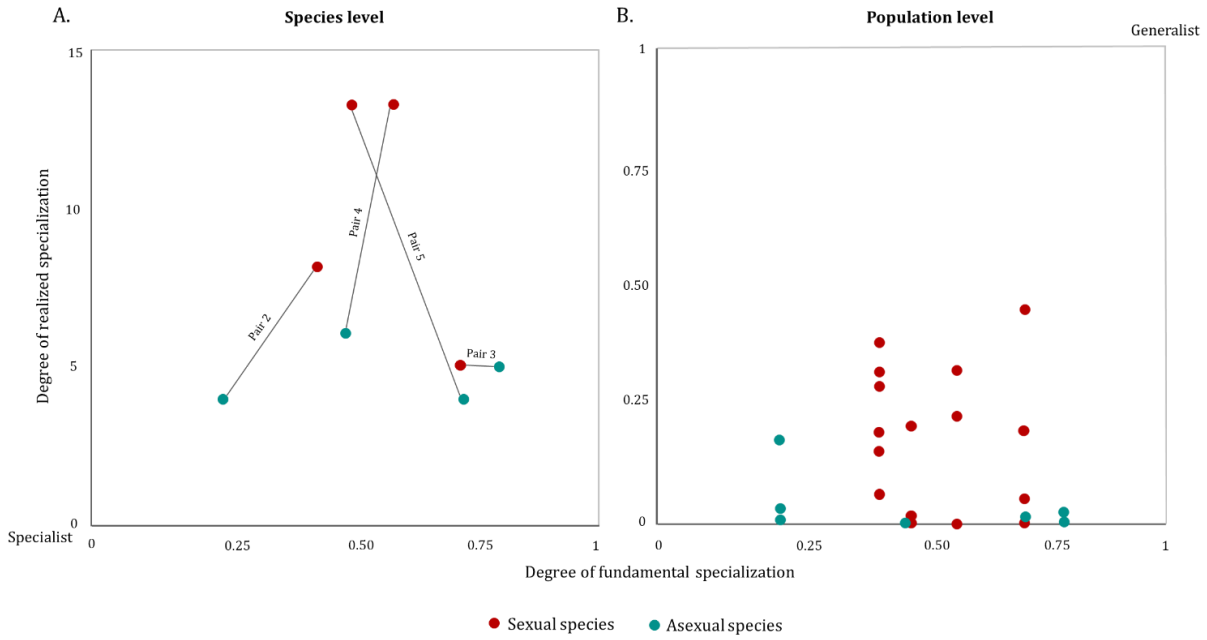

Figure 5. Realized and fundamental feeding niche breadths of sexual and asexual stick insects are not correlated. Shown is the specificity index Tau of the eight *Timema* species of this study, calculated from the weight gain after ten days as a function of the realized feeding niche at the species level (A) or at the population level (B). For species pair numbers, see Fig. 3.

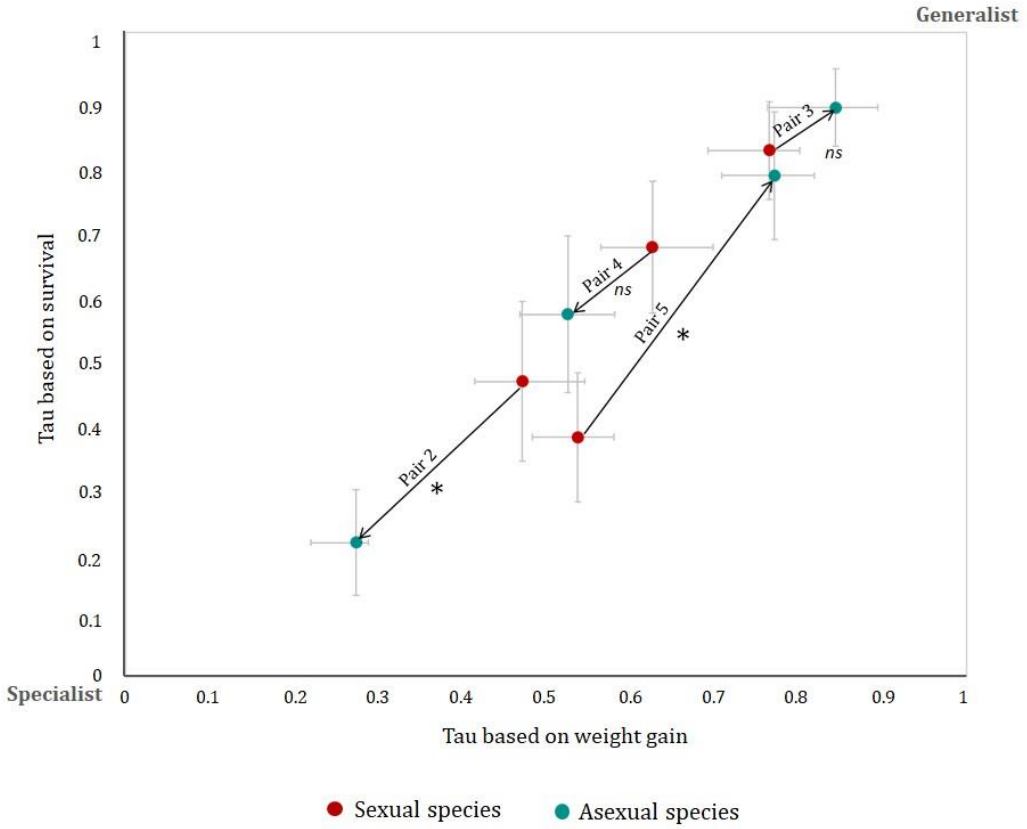

Figure 6. Fundamental feeding niche breadth of sexual and asexual stick insects. Shown are the specificity indices Tau based on weight gain and survival during ten days. Stars indicate significant differences of the Tau indices of the sexual and asexual species of a pair. For species pair numbers, see Fig. 3.

Finally, we found that the fundamental feeding niche breadths were not correlated with the size of their realized feeding niche, neither at the species level (Pearson's correlation; r= 0.13, p= 0.77; Fig. 5A), nor at the population level (r= -0.14, p= 0.50; Fig. 5B).

DISCUSSION

In this study we investigated if sexual and asexual stick insect species and populations differ in their realized feeding niches and how such differences come about. We find that asexuals have broad niches, with *Timema* asexuals generally featuring smaller realized feeding niches than their sexual counterparts. Specifically, in four out of five sexual-asexual *Timema* species pairs, sexuals use about twice as many plants as the asexuals. In the fifth species pair, *T. poppensis*/*T. douglasi,* sexuals and asexuals use the same number of host plants. This species pair is likely an exception to the general pattern in *Timema* because of their ability to utilize redwood as a hostplant. We have shown in a previous study that sexual *Timema* species adapted to this specific host plant are ecologically highly specialized (Larose et al., *in review*). This high level of ecological specialization in the sexual makes further specialization in the related asexual relatively unlikely.

To develop insights into how the comparatively narrower realized niches of asexual versus sexual *Timema* species come about, we quantified the size of their fundamental feeding niches. This allowed us to test if the size of the fundamental niche constrains the size of the realized niche, i.e., whether the reduced realized niche size in asexuals results from a reduced fundamental niche size. Fundamental feeding niche size varied significantly among all *Timema* species, however there was no overall difference between reproductive modes. Fundamental niche size therefore does not explain why sexuals have broader realized niches than asexuals in *Timema*. Specifically, in two species pairs the estimated fundamental niche size was very similar for sexuals and asexuals. In the other two pairs, fundamental niche was different between sexuals and asexuals, however the direction differed; In one species pair (*T. cristinae*/*T. monikensis*) the asexual species had

a narrower fundamental niche than the sexual one, in the other (*T. podura/T. genevievae*) the asexual species had a broader fundamental niche than the sexual one. The latter case is particularly interesting because *T. genevievae* is a very old asexual lineage (~1.5-2 myr) and the oldest asexual *Timema* known (Schwander *et al.* 2011). The broad fundamental feeding niche in *T. genevievae* is consistent with predictions from the *General Purpose Genotype* (GPG) theory, which posits that clones with broad environmental tolerances (i.e., broad fundamental niches) should be selectively favored as such clones would be characterized by low variance in fitness across environments (Lynch 1984; Fig. 2).

Our population level estimates of niche breadth in asexuals should be largely equivalent to clone level measurements given that genotypic diversity in asexual *Timema* populations is extremely low (Bast *et al.* 2018). Thus, the "*General purpose genotype*" hypothesis in *T. genevievae* could help explain why this asexual lineage has survived for so long in the absence of sex. General purpose genotypes are also believed to contribute to the persistence of one of the oldest known asexual species, the darwinulid ostracod *Darwinula stevensoni*, which has probably existed as an obligate asexual for 25 million years (Straub 1952). It shows almost no morphological (Rossetti & Martens 1998) or genetic (Schön *et al.* 1998) variability, yet it is a very common and cosmopolitan species (Griffiths & Butlin 1994) with broad tolerances for salinity and temperature (Van Doninck *et al.* 2002).

In contrast to the old asexual *T. genevievae*, our findings in the youngest studied *Timema* asexual, *T. monikensis,* are consistent with the *Frozen niche variation* model (FNV). This model suggests that the phenotypic distribution (i.e., fundamental niche) of a young, recently derived asexual lineage will be narrower than that of its genetically variable

sexual ancestor (Vrijenhoek 1984; Fig. 1). Indeed, *T. monikensis* is the only studied asexual that features a narrower fundamental niche than its sexual relative *T. cristinae* (Figs. 3 & 6).

Given asexual *Timema* do not generally have narrower fundamental niches than sexual *Timema*, the narrow realized niches in asexuals are likely a consequence of biotic interactions that affect niche size in asexuals more strongly than in sexuals. A likely biotic factor affecting realized niches in *Timema* is selection imposed by predators (e.g., Sandoval 1994a, b; Nosil *et al.* 2003; Nosil 2004). Several *Timema* species feature a natural color polymorphism conferring crypsis on different host plants (Sandoval 1994a; Sandoval 1994b) and we therefore tested for links between color polymorphism, realized niche size and reproductive mode in *Timema*. The sister species *T. douglasi* and *T. poppensis* do not feature any color polymorphism, but in the four remaining species pairs, intra-population color polymorphism is always higher in the sexual than asexual species. However, the level of polymorphism was only correlated to the size of the realized niche at the species level, not at the population level. Nevertheless, this higher degree of color polymorphism in sexuals may allow for reduced predation rates on a larger number of plants relative to asexuals, potentially explaining the narrower realized niche size in asexual species.

Two meta-analyses (Ross *et al.* 2013; Van Der Kooi *et al.* 2017)have previously investigated realized niche size in relation to reproductive mode. Contrary to our results, both of these meta-analyses find that asexual species have broader realized niches than sexual species. Both studies highlight that this is most likely due to the fact that sexual species with broad niches and large geographic ranges are characterized by large

population sizes and are therefore more likely to generate asexual linages. Because of this, disentangling the effects of geographic range, niche breadth, and reproductive mode in these studies is difficult and constrains direct comparison with our work.

In conclusion, we provide the first comparative study of realized and fundamental niches in replicated asexual-sexual species pairs. We found that sexual *Timema* species have a larger realized niche than asexual ones, but this difference is not explained by a similar difference in fundamental niche size. Thus, the smaller realized niches in asexuals are likely a consequence of biotic interactions that constrain asexuals more strongly than sexuals. Evaluating potential links between population-level polymorphism, realized feeding niche size and biotic interactions (especially predation and competition) will be a challenge for future studies. We further found that the oldest asexual lineage is more generalist than it sexual relative such that its broad fundamental feeding niche could help explain its unusually long maintenance in the absence of sex.

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SUPPORTING INFORMATION

Table S1. Color morphs of ten *Timema* species

	Color morphs[1]													
***Timema* species**	**b**	**bl**	**br**	**db**	**dg**	**g**	**gr**	**oc**	**ol**	**sg**	**sgp**	**r**	**y**	**w**
T. bartmani			x			x	x		x	x	x			
T. tahoe			x			x				x	x			
T. cristinae	x			x		x	x	x		x		x	x	
T. monikensis	x			x		x							x	
T. poppensis						x							(x)	
T. douglasi						x							(x)	
T. californicum	x	(x)				x	x					x	x	(x)
T. shepardi						x							(x)	
T. podura	x		x	x	x	x			x					
T. genevievae					x									

[1]For morph name abbreviation, we used: b, beige; bl, blue ; br, brown, db, dark brown, dg, dark grey; g, green; gr, grey; oc, ocre; ol, olive; sg, striped green; sgp, stripped green with pink head; r, red; y, yellow;w, white

A cross indicates that morph has been observed in a given species. A cross in brackets indicates that the morph was observed at a frequency of less than 0.5% in a given species, all populations combined.

Table S2. Details about the populations of nine *Timema* species sampled. Number of individuals refer to the total number of individuals sampled in these locations on different host plants.

Timema species	Location name (GPS coordinates)	Number of individuals per host plant sampled[1]	Morph frequency[2] per sampling location
T. bartmani	YMCA (34°09'48.8"N 116°54'22.6"W)	0 oak, 0 pin, 350 wf	17%br, 34%g, 44%gr,5%ol
	Jenks lake (34°09'55.1"N 116°52'56.4"W)	1 ced, 12 pin, 65 wf	8%br, 23%gr, 4%ol, 12%, 38%sg, 15%sgp
T. tahoe	Bliss (38°58'31.9"N 120°05'58.6"W)	0oak, 0pin, 72 wf	5%br, 32%gr, 36%sg, 27%sgp,
	Vista (38°45'34.8"N 120°11'57.2"W)	0oak, 0pin, 51wf	23%gr, 56%sg, 21%sgp
	SN (39°03'33.0"N 119°56'40.0"W)	0oak, 0pin, 26wf	57%sg, 43%sgp
T. cristinae	Ojai1 (34°31'01.7"N 119°16'39.7"W)	245 lil, 73 mah, 6 mz, 70 oak, 5 toy	2%b, 4%db, 87%g, 5%r, 1%y
	Ojai2 (34°30'20.0"N 119°16'47.5"W)	23lil, 62 mah, 11 mz, 28 oak	3%b, 3%db, 69%g, 25%r
	Ojai3 (34°31'59.6"N 119°14'51.8"W)	8 cha, 2 lil, 20 mah, 8 oak	9%b, 6%db, 59%g, 6%gr, 4%r, 9%sg, 7%y
	WTA1 (34°30'46.6"N 119°46'41.7"W)	597 cha, 317 mah, 78 oak	3%b, 10%db, 23%g, 3%oc, 61%sg <1%y
	WTA2 (34°30'22.3"N 119°46'05.3"W)	81 cha, 1 mah, 8 mz, 9 oak, 2 toy	3%b, 10%dgb 21%g, 2%oc, 64%sg
	WTA3 (34°30'56.8"N 119°46'43.7"W)	60 cha, 24 lil, 5 mz, 7 toy	2%db, 21%g, 7%oc, 69%sg, 1%r
	WTA4 (34°29'58.3"N 119°43'08.2"W)	100 cha, 253 mah, 2 oak	4%b, 7%db, 80%g, 2%oc, 7%r
T. monikensis	Sycamore (34°06'33.7"N 118°54'51.0"W)	0 cha, 9 lil, 0 oak, 0 rdw	<1%b, 5%db, 95%g
	For Sale (34°06'53.6"N 118°51'11.3"W)	0 lil, 0 oak,13rdw	9%b, 13%db, 69%g, 9%y
	Decker (34°06'10.6"N 118°51'42.4"W)	12 lil, 0 mz, 0 oak, 0 rdw	23%b, 35%db, 42%g
T. poppensis	Fish Rock (38°49'05.1"N 123°35'03.5"W)	137 df, 0 lil, 14 rdw	100%g
	Fish Rock2 (38°54'57.1"N 123°18'00.6"W)	34 df, 0 oak, 32 rdw	100%g
	Bear Creek (37°09'56.2"N 122°00'56.4"W)	85 df, 0 oak, 35 rdw	100%g
	Madonna (37°01'07.5"N 121°43'32.0"W)	0 mz, 0 oak, 403 rdw	100%g
T. dougasi	Orr Springs 1 (39°12'44.5"N 123°18'30.2"W)	42 df, 0 cha, 2 mz, 0 oak	99.99%g, 0.01%y
	Manchester 12 (38°58'57.2"N 123°28'10.4"W)	1073 df, 5 mz, 0 oak	100%g
T. californicum	Skyline (37°14'43.6"N 122°06'37.0"W)	2 cha, 18 mz, 43 oak	8%b, 89%g, 3%r
	Saratoga (37°11'47.0"N 122°02'27.1"W)	4 cha, 12 mz, 4 oak	12%b, 82%g, <1%gr, 4%r, 1%y
	Summit (37°02'43.2"N 121°45'11.6"W)	51 mz, 0 oak, 0 rdw	10%b, 2%bl, 85%g, 3%r
T. shepardi	Elk (39°16'42.2"N 122°55'39.6"W)	0 lil, 304 mz, 0 pin, 0 oak	100%g
	Manchester 2	0 df, 30 mz, 0 oak	100%g

	(38°57'22.4"N 123°32'04.9"W)		
	Orr Springs 2 (39°12'02.2"N 123°17'38.1"W)	1df, 0 lil, 200 mz	99.99%g, 0.01%y
T. podura	Indian (33°47'50.5"N 116°46'35.5"W)	79 cha, 60 lil, 0 mah, 7 mz, 0 oak	5%b, 36%db, 24%dg, 23%g, 2%oc, 10%ol
	Poppet (33°51'36.9"N 116°50'20.4"W)	45 cha, 0 lil, 0 mz	4%b, 14%db, 14%dg, 45%g, 23%ol
T. genevievae	HW20 (38°59'38.4"N 122°31'26.4"W)	60 cha, 0 mz, 0 oak	100%dg
	Antonio (37°19'42.0"N 121°29'07.6"W)	248 cha, 0 mah, 0 oak	100%dg

[1]For plant name abbreviations, we used: ced, insense cedar (*calocedrus decurrens*); cha, chamise (*adenostoma fasciculatum*); df, douglas fir (*pseudotsuga menziesii*); lil, califonian lilac (*ceanothus spp*); mah, montain mahogany (*cercocarpus betuloides*); mz, manzanita (*arctostaphylos spp*); oak, oak (*quercus spp*); pin, pinus (*pinus spp*); rdw, redwood (*sequoia sempervirens*); toy, toyon (*heteromeles arbutifolia*).
[2]For morph name abbreviation, see table S1

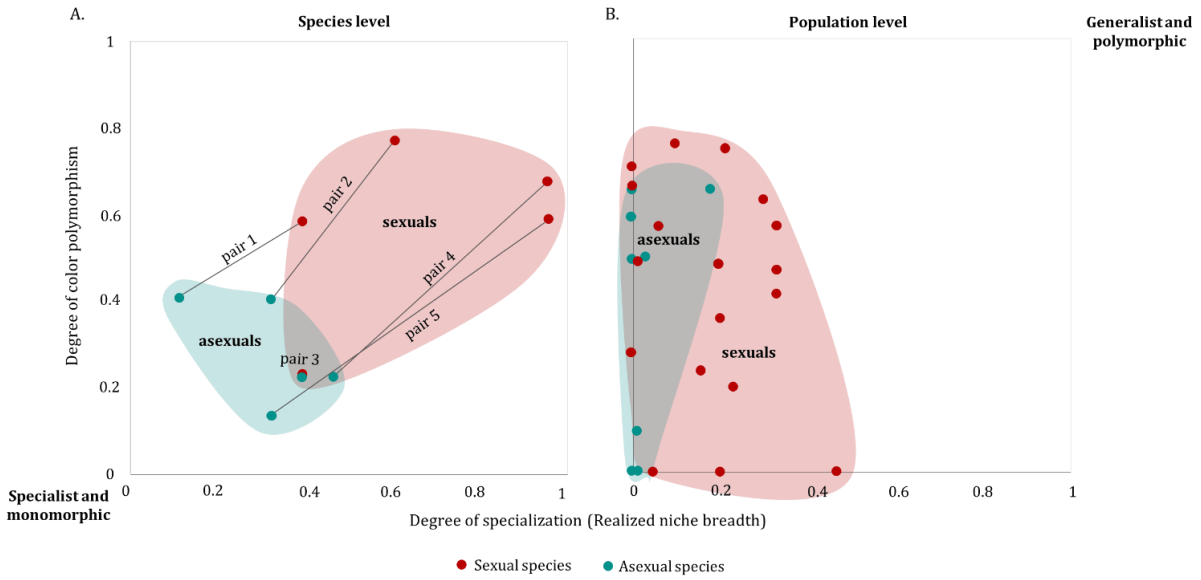
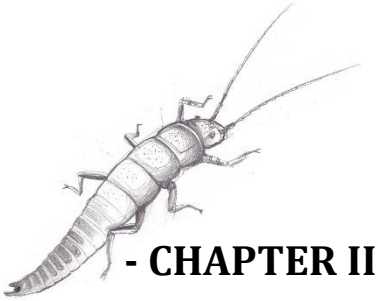


Figure S1. Correlation between color polymorphism and realized feeding niche breadth of Timema at the species (A) and at the population (B) levels. At the species level (A), the polymorphism levels and realized feeding niche sizes are estimated from a count of the different color morphs and of the known host plants in each species respectively. At the population level (B), the polymorphism level is estimated using the inverse Simpson diversity index, and the realized feeding niche size is estimated using the Tau index. In this case, 0 corresponds to specialism and monomorphism, and 1 corresponds to generalism and extreme polymorphism.



- CHAPTER III -

Nematode endoparasites do not codiversify with their stick insect hosts

Running head:
No host-endoparasite co-diversification

C. Larose and T. Schwander

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Author Contributions: TS collected the data. CL analysed the data. CL wrote the first draft of the manuscript, and TS provided substantial input and revisions.

ABSTRACT

Host-parasite coevolution stems from reciprocal selection on host resistance and parasite infectivity and can generate some of the strongest selective pressures known in nature. It is widely seen as a major driver of diversification, the most extreme case being parallel speciation in hosts and their associated parasites. Here, we report on endoparasitic nematodes, most likely members of the mermithid family, infecting different *Timema* stick insect species throughout California. The nematodes develop in the haemolymph of their insect host and kill it upon emergence, completely impeding host reproduction. Given the direct exposure of the endoparasites to the host's immune system in the haemolymph, and the consequences of infection on host fitness, we predicted that divergence among hosts may drive parallel divergence in the endoparasites. Our phylogenetic analyses suggested the presence of two differentiated endoparasite lineages. However, independently of whether the two lineages were considered separately or jointly, we found a complete lack of co-divergence between the endoparasitic nematodes and their hosts in spite of extensive genetic variation among hosts and among parasites. Instead, there was strong isolation by distance among the endoparasitic nematodes, indicating that geography plays a more important role than host-related adaptations in driving parasite diversification in this system. The accumulating evidence for lack of co-diversification between parasites and their hosts at macro-evolutionary scales contrasts with the overwhelming evidence for co-evolution within populations and calls for studies linking micro- vs macro-evolutionary dynamics in host-parasite interactions.

Keywords: co-diversification, cophylogeny, endoparasite, host-parasite interaction, *Timema* stick insects, Mermithid nematodes.

INTRODUCTION

Parasites are ubiquitous in nature and are known to play a fundamental role in community ecology and the evolution of the hosts they infect (e.g., Thompson, 1994; Bohannan & Lenski, 2000; Woolhouse *et al.*, 2002; Decaestecker *et al.*, 2005; Schmid-Hempel, 2011). By definition, parasites have a negative effect on host fitness, favoring selection of enhanced defence or resistance mechanisms in the hosts. In turn, host defence mechanisms are generally detrimental for parasites, leading to selection for counter-adaptations in the parasites. Host-parasite coevolution thus stems from reciprocal selection on host resistance and parasite infectivity (e.g., Thompson, 1994; Clayton *et al.*, 1999; Carius *et al.*, 2001; Dybdahl *et al.*, 2014). Evidence that coevolutionary interactions drive evolutionary changes stems from taxonomically diverse host systems, including bacteria (e.g., Weitz *et al.*, 2005), plants (e.g., Dodds & Rathjen, 2010; Karasov *et al.*, 2014), invertebrates (e.g., Ebert, 2008; Decaestecker *et al.*, 2007), and vertebrates (Kerr, 2012). As a consequence, host-parasite coevolution is widely seen as a major driver of diversification, the most extreme case being co-diversification or parallel speciation in hosts and their associated parasites (e.g., Clarke, 1976; Price, 1980; Kiester *et al.*, 1984; Buckling & Rainey, 2002; Thompson *et al.*, 2005; Yoder & Nuismer, 2010; Ricklefs, 2010; Weber & Agrawal, 2012; Masri *et al.*, 2015).

Co-diversification is particularly expected for endoparasites (more than for ectoparasites) given their direct interaction with the host immune system (Poinar Jr, 1974; Poulin, 2007; Cressler *et al.*, 2014, 2015). Here, we report on endoparasitic nematodes which infect different species of stick insects in the genus *Timema*. *Timema* are herbivorous, wingless stick insects native to the western part of the United States

(Vickery, 1993). We discovered endoparasitic nematodes serendipitously when collecting *Timema* stick insects in the field; an individual nematode larva occasionally emerged from a *Timema* host, killing its host in the process. This parasitic infection thus induces a dramatic cost on host fitness. We presumed that these nematodes belong to the Mermithidae family, given their ecology (Poinar *et al.*, 1976) and morphology (Poinar, 1975). Mermithid nematodes are mainly known as endoparasites of insects (Kaiser, 1991; Nikdel *et al.*, 2011), and occasionally of other invertebrates (Vandergast & Roderick, 2003). Their life cycles vary among species, but females of terrestrial species typically lay eggs in the soil during periods of high moisture. Preparasites (corresponding to larval stage four) then hatch from eggs and migrate to the surface in search of a suitable host. When a preparasite finds a host, it enters the host's hemocoel through a hole pierced into the cuticule and develops in the hemocoel while feeding from the hemolymph (Poinar *et al.*, 1976; Colbo, 1990). The fully developed mermithid larvae then emerge through the intersegmental joints of the host, killing the host in the process. After emergence, the free-living, non-feeding postparasites burrow in the soil where they molt to the adult stage, mate and lay eggs (Poinar & Otieno, 1974).

We found mermithid-like endoparasitic nematodes in nine different *Timema* stick insect species, which prompted us to test for co-diversification of these nematodes and their hosts. We infer the most probable evolutionary events that have generated the present distribution of parasite lineages among the different host species. This allows us to test whether adaptation to different host species has contributed to endoparasite diversification.

METHODS

Sample collection and molecular methods

Timema stick-insects from 13 different species (Fig. 1.a) were collected throughout California, between 2007 and 2015, in order to perform a number of experiments not related to the present study. While maintaining stick insects in the laboratory, we occasionally found parasitic nematodes that emerged from an individual female stick insect, killing its host in the process. Females from which the nematodes emerged died before producing a single egg and had undeveloped ovaries, indicating that these nematodes completely impede reproduction of their host. Each emerged nematode was collected and stored in 95% alcohol until further use. Even though thousands of stick insects were collected over the nine years, we only assembled a set of 31 nematodes from nine of the 13 sampled *Timema* stick insect species, with a nematode emerging from 0 to 1.2% of host individuals, depending on years and host species. These emergence rates only consider nematodes that successfully developed within their hosts and do not take into account cases where nematode development would have been suppressed by the host's immune system. Furthermore, given the size of the nematodes (Fig. 1.b), undetected emergences among the collected stick insects are highly unlikely, an assumption confirmed by the dissection of 821 stick insects of which fewer than 1% were infected (2 out of 821). We therefore tested for host-parasite coevolution between the endoparasitic nematodes and their *Timema* hosts with multiple nematodes available for four host species. For one of these, the most intensively sampled host (*T. cristinae*), we had 16 nematodes, of which we used nine for our study (three from each of three different host populations), for a total of 24 nematodes from nine different *Timema* species (Fig 1.a).

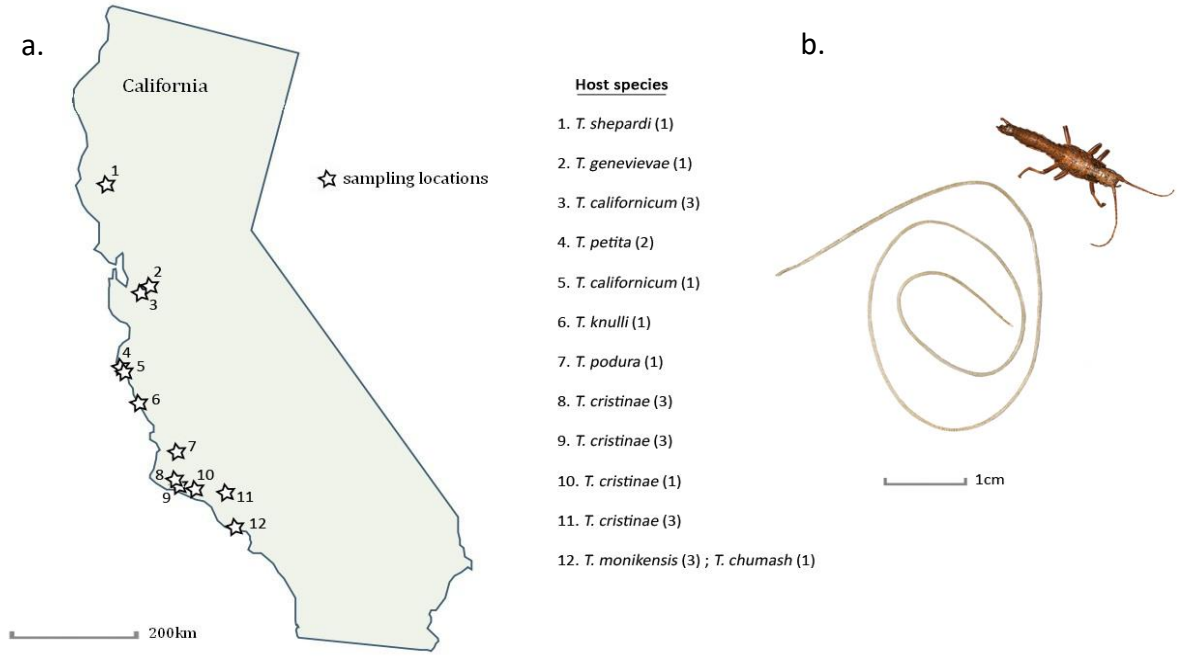

Figure 1. a. Locations of the endoparasitic nematodes sampled in this study. Numbers in brackets indicate the number of nematodes per host species and location. Please note that the large number of nematodes collected from the *T. cristinae* host is explained by *T. cristinae* being the most intensively sampled host species (not by this species being more infected than others). **b. Picture of an endoparasitic nematode** after it exited and killed its *Timema* host.

DNA from the nematodes was extracted using a Quiagen DNeasy Blood & Tissue kit following the manufacturer's protocol. We used two primer pairs from other studies for amplifying a ~1200 bp portion of the 18S small ribosomal subunit (18S rRNA): the universal SSU primers SSU18A (5'-AAAGATTAAGCCATGCATG) and SSU26R (5'-CATTCTTGGCAAATGCTTTCG) from Blaxter *et al.* (1998) and 18S-5F (5'-GCGAAAGCATTTGCCAAGAA) and 18S-9R (5'-GATCCTTCCGCAGGT TCACCT) from Vandergast and Roderick (2003). PCR reactions were performed in 25 µl containing 0.5U AmpliTaq DNA Polymerase (Applied Biosystems, Foster City, CA), 1.8mM MgCl2, 0.2mM each dNTP, and 0.4mM each primer. For both primer pairs, a touchdown PCR protocol

was employed. The first 10 cycles were performed with denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and an extension of 40 s at 72 °C. Ten additional cycles were run with an annealing temperature of 50°C and the 20 final cycles with an annealing temperature of 45°C. Ten min final extension at 72°C ended the amplification. PCR products were visualized on agarose gels stained with ethidium bromide. Five µl of each PCR product were purified using 4µl of ExoI (20U/µl) (Thermo Scientific) mixed with FastAP Thermosensitive Alkaline Phosphatase (1U/µl) (Thermo Scientific). After addition of 5 µl (5mM) forward primer, purified PCR products were sent to *GATC Biotech, Germany (www.gatc-biotech.com)* for Sanger sequencing. We aligned the 18S rRNA portions using the algorithm MUSCLE (Edgar, 2004) as implemented in SeaView 4.5.4 (Galtier *et al.*, 1996; Gouy *et al.*, 2010). The final alignment consisted of 1078 bp (including 7-26 bp gaps). GenBank accession numbers are indicated in Table S1.

Phylogenetic placement of the endoparasitic nematodes

To verify that the *Timema* endoparasitic nematodes indeed belong to the Mermithidae family, we built a maximum likelihood phylogeny using the newly generated 18S rRNA sequences and published sequences from Ross *et al.* (2010). The published sequences were chosen to represent the four nematode clades proposed by Blaxter *et al.* (1998), which are known to comprise endoparasitic nematodes ("Clades I, III, IV and V", see Fig. 2.c). For the first clade ("Clade I" in Blaxter *et al.*, 1998), which includes the Mermithidae family (Fig. 2.b), we used 24 sequences. Three representative sequences per clade were used from the three remaining clades ("Clade III" to "Clade V" in Blaxter *et al.*, 1998), for a total of 33 sequences. Details for each sequence, including GenBank accession numbers, are shown in table S1. Using likelihood scores as implemented in FindModel (Posada & Crandall, 1998; Tao *et al.*, 2005), we inferred that the GTR+G model best described our

dataset (LnL = -6947, AIC = 13912). We used this model to construct a maximum likelihood tree in SeaView 4.5.4 (Galtier *et al.*, 1996; Gouy *et al.*, 2010) with heuristic searches (excluding gaps). The bootstrap support for each branch was calculated using the same model with 1000 replicates.

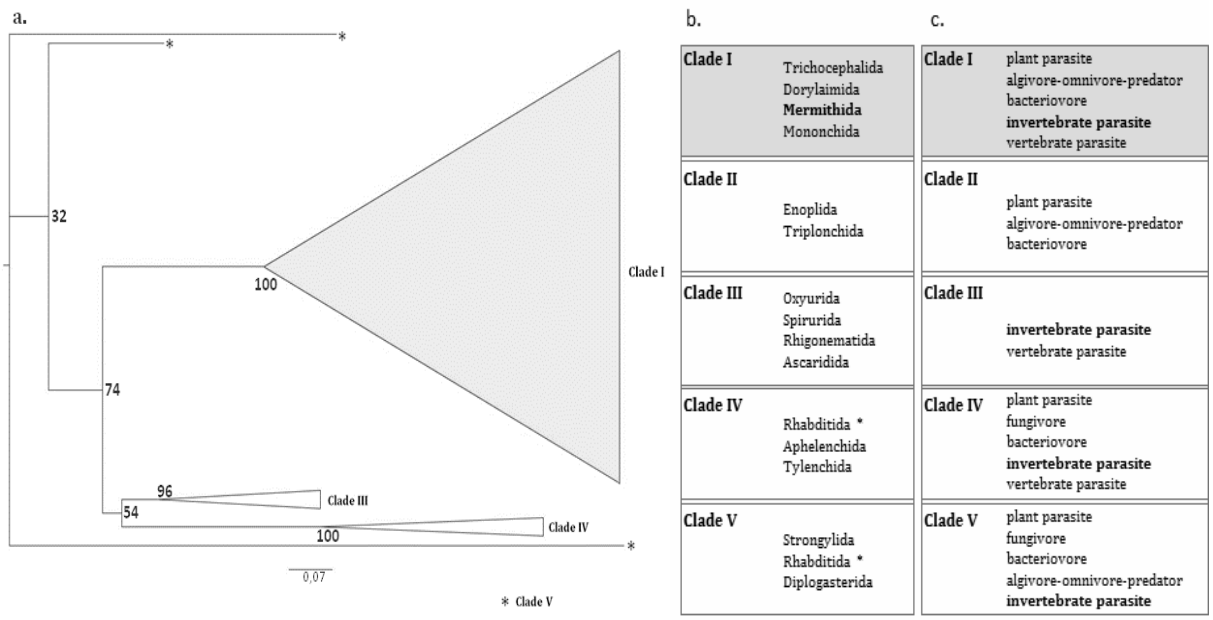

Figure 2. Phylogenetic placement of endoparasitic nematodes from *Timema* within the Nematoda phylum. (a) Maximum likelihood phylogeny based on the 18S rRNA sequence of 57 nematodes. The highlighted group corresponds to Clade I, which comprises the 24 *Timema* endoparasitic nematode sequences (see Fig 3 for details of this clade). Numbers associated with branches indicate bootstrap support (1000 replicates). (b) Nematode orders described in each clade and (c) their trophic ecologies. Information indicated in (b) and (c) are from Blaxter *et al.* (1998).

We also tested whether *Timema* endoparasites are closely related to the *Clitarchus* stick-insect endoparasite found by Yeates and Buckley (2009) by adding the 18S sequence portion from that species to the sequence set described above and running the same phylogenetic analyses. However, because the *Clitarchus* 18S sequence portion was much

smaller (781 bp) than the amplified portion in *Timema* (1200bp) and thus less informative, we did not use this sequence for any further analyses.

Host-parasite co-phylogenetic analyses

We used two co-phylogeny methods to infer the most probable co-evolutionary history between *Timema* and their endoparasitic nematodes: the method implemented in the program TreeMap 3.0β (Page, 1994; Charleston, 1998, 2002), and the one implemented in Jane 4.0 (Conow *et al.*, 2010). Both methods reconcile tree topologies of hosts and parasites by inferring four or five (depending on the method) evolutionary events: 1) "Co-divergence", which occurs when the host and parasite diverge simultaneously; 2) "duplication", which corresponds to the divergence of the parasite, with both descendants of the parasite lineage remaining associated with the same host; 3) "host-switch", which is a duplication followed by the shift of one parasite lineage to a new host; 4) "parasite loss", which corresponds to the apparent absence of a parasite lineage in the descendants of a host that previously had an associated parasite, and 5) "failure to diverge", which occurs when a host speciates but the parasite does not (the same parasite remains on both new host species). Each of these evolutionary events is given a cost related to the likelihood of that event (Ronquist, 1997), and co-phylogenetic tree reconciliation then identifies the combination of events that generates the observed host and parasite phylogenies while minimizing the total costs.

The TreeMap 3.0β method considers four of the five events described above (co-divergence, duplication, host-switching, and parasite loss), and finds the best cost scheme settings while maximizing the probability of co-divergence (i.e., minimizing costs assigned to co-divergence). It then infers the maximum number of co-divergence events

and the minimum number of non co-divergence events needed to reconcile the observed host and parasite phylogenies (see Charleston, 1998 for the details of the tree-mapping method). Finally, TreeMap 3.0β graphically depicts the differences between host and parasite phylogenies in a “tanglegram” (Page, 1994, 1995).

The Jane 4.0 method performs the reconciliation analyses using all five described evolutionary events, whereby the cost of each event is chosen depending on the biological system (see Conow *et al.*, 2010 for the details of the tree-mapping method). It has been shown that the outcome of event-based analyses is heavily dependent on the cost scheme employed (Merkle *et al.*, 2010), and choosing a biologically meaningful cost scheme *a priori* is often difficult (De Vienne *et al.,* 2013). To ensure we would not fail to detect co-speciation because of an inappropriate cost scheme, reconciliation of the host and parasite phylogenies was performed using three different types of cost schemes (see also results Table 1). In the first type, referred to as "equal", all events were of equal cost. The second type of cost-schemes ("co-divergence maximization") maximized the probability for obtaining co-divergence by assigning a low cost to co-divergence events as suggested by Charleston (2002) and Hendricks *et al.* (2013). Finally, the third type of cost-schemes, called "alternatives", was used to find scenarios generating good (i.e., low cost) tree reconciliations. In these alternatives, we no longer tried to maximize the probability of co-divergence, and instead varied the relative costs associated with co-divergence, duplication and host-switch events to obtain evolutionary scenarios with a good fit to the observed data. Other than the cost schemes, we used default settings for all Jane 4.0 parameters as recommended by Conow *et al.* (2010), with the number of generations (G = 300) set as two times higher than the population size (S = 150). Varying the default settings did not affect our results (data not shown).

Statistical significance of the inferred evolutionary scenarios is evaluated differently in the TreeMap 3.0β *vs* Jane 4.0 methods. To test whether the number of observed co-divergence events between hosts and parasites is greater than expected by chance, TreeMap 3.0β generates 1000 random parasite trees. The reported p-value then corresponds to the proportion of random parasite trees that result in the same number, or more, co-divergence events than the observed parasite tree (Page, 1990, 1994). We also tested whether distances (branch lengths) in associated subtrees of the parasite and the host trees were significantly correlated, as would be expected under co-divergence.

In contrast to TreeMap 3.0β, Jane 4.0 estimates the observed total cost for the most parsimonious scenario of host-parasite tree reconciliation (under a given cost scheme). The goodness-of-fit of this scenario is then evaluated by calculating the total costs for the most parsimonious host-parasite tree reconciliations obtained from each of 1000 randomly generated parasite trees (Conow *et al.*, 2010).

Both TreeMap 3.0β and Jane 4.0 use the phylogenies of hosts and their parasites as input. To perform the cophylogenetic analyses implemented in TreeMap 3.0β, we used a robust, previously published *Timema* phylogeny (Schwander *et al.*, 2011, Schwander *et al.*, 2013), which includes host species for which we did not find any parasites during nine years of sampling. Because hosts without associated parasites cannot be used in Jane 4.0, we pruned the host phylogeny to comprise only the nine *Timema* species for which we found parasites in analyses with Jane 4.0.

Finally, we also assessed whether geographic distance could contribute to divergence among endoparasites. Pairwise genetic divergences among nematodes were estimated from p-distances (gaps deleted) in MEGA 6.0 (Tamura *et al.*, 2013). Genetic differentiation due to isolation by distance among endoparasitic nematodes was assessed by conducting Mantel tests in XLSTAT (Addinsoft Version 2015.3.01.19251).

RESULTS

Phylogenetic placement of the endoparasitic nematodes

The maximum likelihood phylogeny confirmed that the *Timema* endoparasitic nematodes are indeed closely related to species from the family Mermithidae of Nematoda (Clade I, Fig. 2.a), and are closely related to the single mermithid ever collected from another stick insect (*Clitarchus*; Yeates and Buckley, 2009; Fig. S1). However, identification of nematodes to family levels is difficult, even with DNA evidence. Moreover, the *Timema* nematodes seem to consist of two distinct lineages, although with little bootstrap support (Fig. S1). To take this apparent phylogenetic structure into account, all the following analyses were applied to either the complete set of nematodes (both lineages combined), or by considering the lineages separately.

Host-parasite cophylogenetic analyses

A visual inspection of the *Timema* host and endoparasite trees does not suggest any coevolution between *Timema* stick insects and their endoparasitic nematodes. This is the case independently of whether the two nematode sub-lineages are analyzed separately or together (see tanglegrams Fig. 3). Indeed, neither the method implemented in TreeMap 3.0β, nor the one implemented in Jane 4.0, provided evidence for coevolution between *Timema* hosts and their parasites. Using TreeMap 3.0β for the two nematode sub-lineages together, we inferred that the most probable coevolutionary history required 16 co-divergence events and a minimum of 43 non-co-divergence events (23 parasite duplications, 9 host-switches and 11 parasite losses). The 16 observed co-divergence events were not more frequent than expected by chance (1000 randomizations of the parasite tree, p-value = 0.976). Furthermore, branch lengths in associated subtrees of the

parasite and the host tree were not significantly correlated (p-values between 0.22 and 1), in contrast to the pattern expected under co-diversification. When considering the two sub-lineages separately, we detected a maximum of 10 cospeciation events for the first and 9 for the second lineage (with respectively 36 and 21 non-codivergence events). These co-divergence events were not more frequent than expected by chance (p-value = 0.936 and p-value > 0.99).

Similar to the results obtained via the TreeMap 3.0β method, we also found no indication of coevolution between hosts and parasites using the methods implemented in Jane 4.0. Analyzing the two nematode sub-lineages together or separately did not affect the results. All different cost schemes used to infer likely scenarios of host and parasite divergence indicated the absence of a co-evolutionary signal (Table 1). Indeed, neither the "equal" cost-scheme nor the three "co-divergence maximization" cost-schemes identified a scenario that would match the observed host and parasite trees better than random trees (p-values between 0.365 and 0.99; Table 1). Plausible evolutionary scenarios with a significantly (or marginally significantly) better match to the observed than to randomized trees were only observed with the "alternative" cost-schemes (Table 1). Each of the plausible scenarios inferred either 0 or 1 co-divergence events, and 11-22 non co-divergence events (Table 1), indicating, again, the lack of co-diversification of endoparasitic nematodes and their *Timema* hosts.

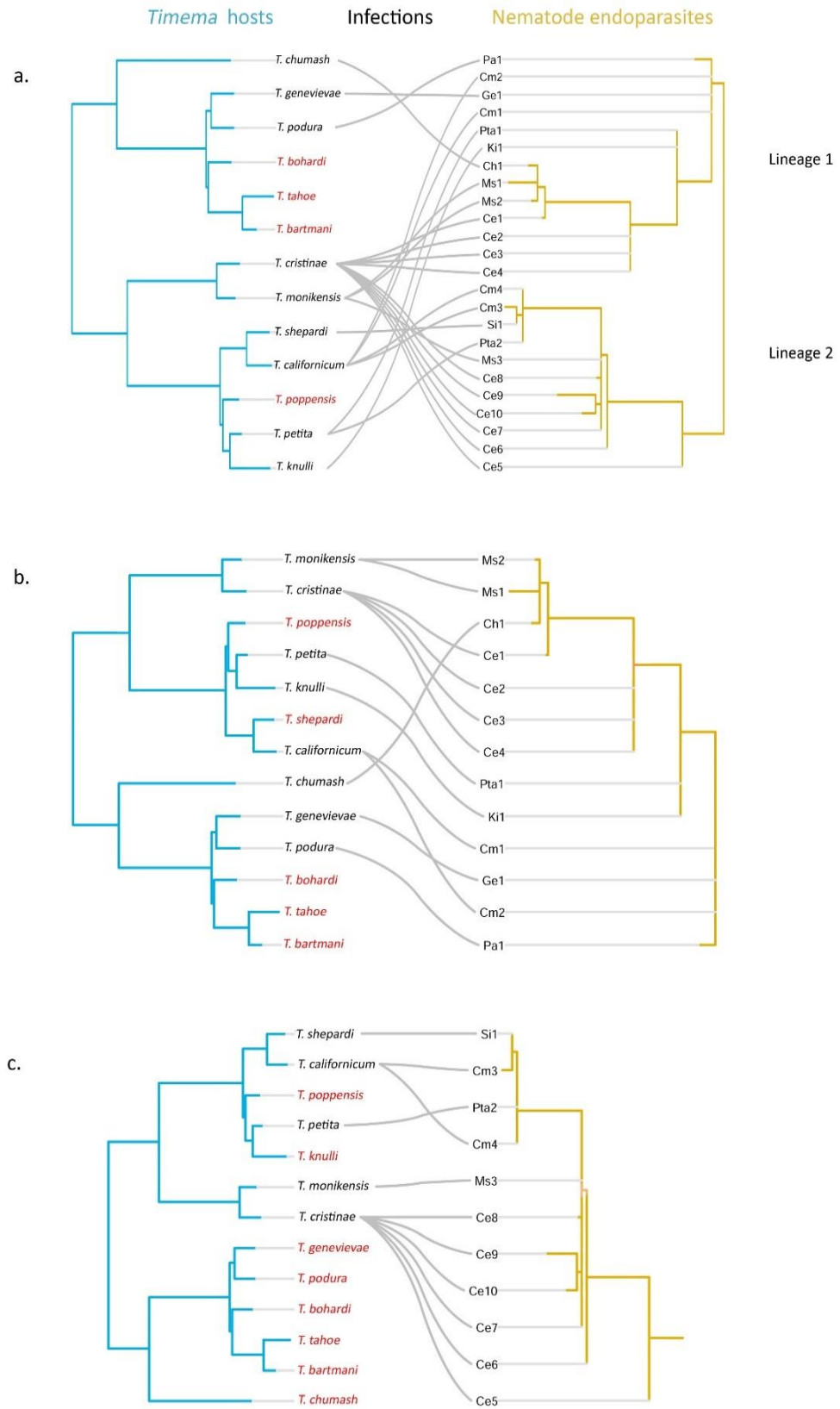

Figure 3. Tanglegrams (generated with TreeMap 3.0β) comparing the nematode endoparasite phylogeny (right) to the *Timema* host phylogeny (left) with grey lines indicating host-parasite associations. The two endoparasitic nematode sub-lineages are combined in (a) and treated separately in (b) and (c).

Table 1. Outcome of cophylogenetic analyses in JANE 4.0, employing different cost schemes.
* Costs are ordered as co-divergence, duplication, host switch, parasite loss, and failure to diverge.
† For each cost scheme, analyses were performed three times: "2lineages" corresponds to the analyses considering both nematode sub-lineages together, while "Lin1" and "Lin2" correspond to the analyses considering only one sub-lineage. Plausible evolutionary scenarios are highlighted in grey.

			Co-divergence	**Non co-divergence**						
Model *Biological interpretation*	**Cost scheme***	**Analyses†**	**Total number of events**	Duplication	Host switch	Parasite loss	Failure to diverge	**Total number of events**	**Total cost**	**P-value**
Equal										
Events of equal costs	11111	2lineages	0	6	17	0	0	23	**23**	1
		Lin1	0	4	8	0	0	12	**12**	1
		Lin2	0	2	8	0	0	10	**10**	1
Co-divergence maximization										
Co-divergence of no cost	01111	2lineages	6	5	12	1	0	18	**18**	0.385
		Lin1	3	3	6	0	0	9	**9**	0.448
		Lin2	2	2	6	0	0	8	**8**	0678
Co-divergence facilitated	-10000	2lineages	9	5	9	9	0	23	**-9**	0.365
		Lin1	4	3	5	7	0	15	**-4**	0.629
		Lin2	4	2	4	4	0	10	**-4**	0.841
Co-divergence facilitated	-11111	2lineages	6	5	12	1	0	18	**12**	0.739
		Lin1	3	3	6	0	0	9	**6**	0.996
		Lin2	2	2	6	0	0	8	**6**	0.708
Alternatives										
Host-switches unlikely	11211	2lineages	1	11	11	0	0	22	**34**	0.115
		Lin1	1	5	6	0	0	11	**18**	**0.068**
		Lin2	0	6	4	0	0	10	**14**	0.310
No host-switches	11N11	2lineages	7	16	NA	36	0	52	**59**	0.133
		Lin1	3	9	NA	27	0	36	**39**	0.610
		Lin2	4	6	NA	8	0	14	**18**	0.100
Maximizing co-divergence, minimizing host-switches	01211	2lineages	5	8	10	2	0	20	**30**	0.231
		Lin1	3	3	6	0	0	9	**15**	0.211
		Lin2	1	5	4	0	0	9	**13**	0.517
Co-divergence and duplication of no cost	00111	2lineages	1	11	11	0	0	22	**11**	**0.094**
		Lin1	1	5	6	0	0	11	**6**	**0.071**
		Lin2	0	6	4	0	0	10	**4**	0.319
Duplication of no cost	10111	2lineages	0	11	12	0	0	23	**12**	0.109
		Lin1	0	5	7	0	0	12	**7**	**0.022***
		Lin2	0	6	4	0	0	10	**4**	0.111

In summary, the lack of a coevolutionary signal in all analyses shows that genetic divergence of the endoparasitic nematodes we collected from *Timema* hosts is not driven by divergence among different host species. Importantly, the lack of a coevolutionary signal between the endoparasites and their hosts is not due to a lack of genetic diversity in the parasites. Indeed, the level of genetic divergence detected among different endoparasites is considerable, with 12% segregating sites and an average sequence divergence of 3.9%.

Timema endoparasites appear to diverge because of geographic separation rather than as a consequence of host-driven divergence. Irrespective of the identity of the host, we observed strong isolation by distance between the endoparasitic nematodes (mantel-test with 10.000 permutations: $r = 0.13$, p-value < 0.0001; Fig. 4a). The pattern was even stronger when both nematode sub-lineages were analyzed separately (partial mantel-test with 10.000 permutations: $r = 0.24$, p-value < 0.0001; Fig. 4b). Indeed, we found genetically similar nematodes parasitizing very distinct *Timema* species (Fig. 3), as nicely illustrated by genetically similar parasites infecting the phylogenetically distinct hosts *T. chumash* and *T. monikensis* at a location where the two hosts co-occur.

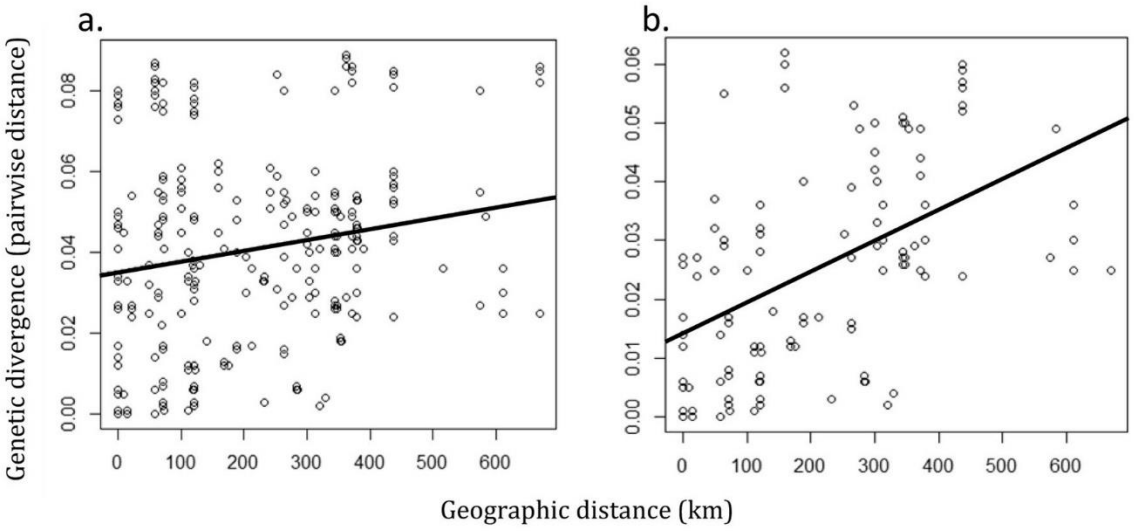

Figure 4. Pairwise genetic distances between endoparasitic nematodes as a function of geographic distances (km) (a) Pairwise distances between sequences from all endoparasitic nematodes (b) Pairwise distances within lineages 1 and 2 (distances between sequences from different lineages are not included).

DISCUSSION

Coevolution, the process of reciprocal adaptation between ecologically interacting species, is considered as a key force generating biological diversity (e.g., Clarke, 1976; Price, 1980; Kiester *et al.*, 1984; Buckling & Rainey, 2002; Thompson *et al.*, 2005; Yoder & Nuismer, 2010; Masri *et al.*, 2015; Ricklefs, 2010). In this study we identified a new group of endoparasitic nematodes, infecting at least nine species of *Timema* stick insects throughout California, as relatives of mermithid nematodes. This is only the second report of mermitid (or mermithid-like) nematodes infecting stick insects, after Yeates and Buckley (2009) found a mermithid nematode infecting a *Clitarchus* stick insect in New Zealand. We found that this mermithid is closely related to *Timema* endoparasites, suggesting few or perhaps even only a single colonization of phasmatodean hosts by mermithids.

In natural *Timema* populations, nematodes emerged from typically less than 1.2 % of the host individuals. Obviously, these low emergence rates only include cases where the parasites have managed to infect the *Timema* hosts and successfully completed their development. They do not take into account the cases where hosts died prior to parasite emergence, or cases where infected hosts managed to suppress parasite development.

The phylogenetic analyses of the endoparasitic nematodes suggested the presence of two sub-lineages. Independently of whether these sub-lineages were considered separately or jointly, and independently of the cophylogenetic analyses conducted (TreeMap 3.0β and Jane 4.0 with a broad range of cost settings), we found a complete lack of co-divergence between the parasites and their *Timema* hosts. We conducted over 30 cophylogenetic

analyses, but the level of congruence between the host and parasite phylogenies was never higher than expected by chance.

It is very unlikely that the absence of host-parasite co-diversification is due to incorrect phylogenies of either the host or the parasite. The *Timema* host phylogeny is very robust (Schwander *et al.*, 2011, Schwander *et al.*, 2013). For the parasite phylogeny, although several nodes are weakly supported, topology errors for the weakly supported nodes would not influence the main result. Indeed, there were many non-codivergence events (Table 1) that concern the well supported nodes in the parasite tree (e.g., nematodes infecting *T. cristinae* hosts, in Fig. 3.a) such that minor topology changes at poorly supported nodes would not change the main conclusion of little or no host-parasite co-diversification.

Similarly, the lack of host-parasite co-diversification is not due to little genetic divergence within the hosts or parasites. Nine different *Timema* species (some of which have diverged for over 20 million years; Sandoval *et al.*, 1998) from a large geographical area (Fig. 1; the two most distant sampling points are separated by 670 km) are infected by these endoparasites. The genetic variation among nematodes is also substantial (average pairwise sequence divergence of 3.9%). Furthermore, we found significant isolation by distance among *Timema* nematodes (Fig. 4). Hence, nematode genetic divergence seems driven much more by geographical separation than by coevolution and adaptation to their hosts, indicating the absence of 'ecological speciation' in this system.

The review of a number of host-parasite systems by Braker (1994) suggested that co-diversification of parasites with their hosts seems to mainly happen when the hosts are

allopatric. This would be the case for *Timema* as there is overall little overlap in the distribution ranges of different *Timema* species (Law & Crespi, 2002). But despite these apparently favorable environmental conditions, we did not find the expected co-diversification.

Similar to the lack of co-diversification between *Timema* hosts and their endoparasitic nematodes, other parasite species known to be strongly host-specific also diverged independently of their host. For example, flatworms in the genus *Lamellodiscus* infect different fish species in *Sparidae* family, with no apparent phylogenetic congruence between the parasites and their hosts (Desdevises *et al.*, 2002). The same observation was made on fish parasitic copepods (Paterson & Poulin, 1999) and trematodes (Cribb *et al.*, 2001). In each of these systems, the lack of co-diversification was suggested to be due to the ecology of the parasites, with short periods outside the hosts, as well as the aquatic environment, which would greatly facilitate parasite dispersal and thus potentially host switches. However, such frequent host switches would be less likely in terrestrial systems like *Timema*. Furthermore, *Timema* are wingless and do not disperse over long distances (Sandoval, 1994; Schwander *et al.*, 2010). As mentioned above, different *Timema* species also feature quite distinct distribution ranges, further constraining the opportunity for host-mediated parasite dispersal and exposure of parasites to alternative hosts species. A notable exception to this general pattern stems from the two distantly related species *T. chumash* and *T. monikensis,* which share a similar nematode parasite strain in the location where these two species co-occur (Fig.1a).

In addition to frequent host switches, several other ecological factors may also contribute to the non-congruence of host and parasite trees. For instance, a number of studies

highlighted the fact that macro-parasites often feature higher mutation rates, smaller effective population sizes and limited dispersal abilities relative to their hosts (e.g. McDonald & Linde, 2002; Criscione & Blouin, 2005; Poulin, 2011). The implications are that genetic drift can be very pronounced in parasites and generate extensive spatial genetic structure independently of divergence among parasite strains infecting different hosts. Drift might indeed be an important mechanism constraining co-divergence of *Timema* endoparasitic nematodes and their hosts. The endoparasitic life cycle, as well as the apparently low frequency of infections in natural stick insect populations (<1.2 %), suggest that the endoparasites' population sizes might be orders of magnitude smaller than their hosts' – unless the same endoparasites also infect non-*Timema* hosts.

A broad host range including species from other genera or even other insect orders could also explain the lack of co-diversification between the endoparasites and *Timema*. Although the ecology and biology of the *Timema* endoparasites have never been studied specifically, the ecology of a range of mermithid nematode species has been well documented (e.g., Poinar, 1975; Poinar *et al.*, 1976; Baker *et al.*, 1998). Mermithid species are typically characterized by strong host specificity (Stoffolano, 1973; Kennedy, 1975; Rohde, 1979, 2002; Noble *et al.*, 1989; Sasal *et al.*, 1998) while the family as a whole is cosmopolitan and infects a broad range of invertebrates (Kaiser, 1991; Vandergast & Roderick, 2003; Nielsen, 2004; Nikdel *et al.*, 2011). Nevertheless it remains possible that some mermithid species are generalists and use a broad range of hosts. A mixture of highly host-specific and generalist species is for example known in parasitoid wasps, which, similar to mermithid nemathodes, kill their hosts at emergence, preventing reproduction of their hosts (see Eggleton and Gaston, 1990 and Godfray, 1994 for a discussion of further similarities between parasitoid wasps and parasitic nematodes).

Future studies on the ecology of the *Timema* endoparasitic nematodes may shed light on these questions.

Thus far, the vast majority of examples revealing strong co-diversification between parasites and their hosts stem from pocket gophers and their chewing lice (e.g. Hafner & Nadler, 1988; Hafner & Page, 1995; Demastes *et al.*, 2002; Hafner *et al.*, 2003) and from swiftlets and their parasitic lice (Page et al. 1998). In both cases, the close relationship between the hosts and their parasites led to identical topologies of the phylogenies, indicating that the hosts and parasites speciated in perfect synchrony (a pattern known as the Farenholz' rule). However, given the accumulating evidence from other host - parasite systems (e.g., see review by De Viennes *et al.*, 2012), including *Timema* and their nematode endoparasites, the pocket gophers/swiftlets - lice systems seem to represent a fairly unusual pattern. Therefore, explaining the frequent lack of co-diversification between parasites and their hosts at macro-evolutionary scales, even though there is a large body of evidence for coevolution between hosts and parasites within populations (micro-evolutionary scale, e.g., Brooks, 1979; Anderson & May, 1982; Kaltz & Shykoff, 1998; Decaestecker *et al.*, 2007) remains a challenge for future studies. Indeed, as previously suggested by De Vienne *et al.* (2012), co-diversification with hosts does not seem to be the predominant mode of speciation in parasites, despite the well-documented occurrence of reciprocal selection over short time-scales. There is thus a crucial need for studies linking micro- vs macro-evolutionary dynamics in host-parasite interactions.

In conclusion, this study reports a new group of endoparasitic nematodes, related to the mermithid family, infecting several species of *Timema* stick insects. We found no co-diversification between these parasites and their hosts, even though co-diversification

might be expected given the close interaction between the parasites and their hosts and the dramatic fitness costs of infection. Instead, geographical distance seems to play a more important role than host-related adaptations in driving genetic differentiation between parasites in this system.

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SUPPORTING INFORMATION

Table S1. Samples iD, host species and sequence information of the nematodes used in this study

Sample iD	Source	Clade (from Blaxter et al., 1998)	Dataset/ Figure	GenBank Accession numbers
Anatonchus tridentatus	GenBank	I	2, S1	AJ966474
Bathyodontus cylindricus	GenBank	I	2, S1	AY552964
Bathyodontus mirus	GenBank	I	2, S1	AY284744
Clarkus papillatus	GenBank	I	2, S1	AY284748
Granonchulus sp.	GenBank	I	2, S1	AY593953
Isomermis lairdi	GenBank	I	2, S1	FN400900
Longidorus elongates	GenBank	I	2, S1	AY687992
Longidorus grandis	GenBank	I	2, S1	AY283165
Mermis nigrescens	GenBank	I	2, S1	AF036641
Mermis sp.	GenBank	I	2, S1	FJ973464
Mermithid sp.	GenBank	I	2, S1	AY284743
Mermithidae	GenBank	I	2, S1	FJ040480
Mononchus truncates	GenBank	I	2, S1	AJ966493
Mononchus tunbridgensis	GenBank	I	2, S1	AY593954
Mylonchulus sp.	GenBank	I	2, S1	AY284761
Mylonchulus arenicolus	GenBank	I	2, S1	AF036596
Paralongidorus maximus	GenBank	I	2, S1	AJ875152
Soboliphyme baturini	GenBank	I	2, S1	AY277895
Trichuris muris	GenBank	I	2, S1	AF036637
Xiphinema bakeri	GenBank	I	2, S1	AY283173
Xiphinema krugi	GenBank	I	2, S1	AY297828
Xiphinema rivesi	GenBank	I	2, S1	AM086673
Xiphinema taylori	GenBank	I	2, S1	AM086676
Xiphinema simile	GenBank	I	2, S1	AM086681
Dirofilaria immitis	GenBank	III	2	AF036638
Raillietnema sp.	GenBank	III	2	DQ503461
Wellcomia slamensis	GenBank	III	2	EF180079
Acrobeles complexus	GenBank	IV	2	AY284671
Bursaphelenchus mucronatus	GenBank	IV	2	AY508022
Strongyloides ratti	GenBank	IV	2	SRU81581
Cephaloboides sp.	GenBank	V	2	AF083027
Rhabditis colombiana	GenBank	V	2	AY751546
Syngamus trachea	GenBank	V	2	AJ920344
Ce1	Current study	I	1, 2, 3, 4, S1	KX301041
Ce2	Current study	I	1, 2, 3, 4, S1	KX301053
Ce3	Current study	I	1, 2, 3, 4, S1	KX301054
Ce4	Current study	I	1, 2, 3, 4, S1	KX301055
Ce5	Current study	I	1, 2, 3, 4, S1	KX301043
Ce6	Current study	I	1, 2, 3, 4, S1	KX301051
Ce7	Current study	I	1, 2, 3, 4, S1	KX301042
Ce8	Current study	I	1, 2, 3, 4, S1	KX301052
Ce9	Current study	I	1, 2, 3, 4, S1	KX301046
Ce10	Current study	I	1, 2, 3, 4, S1	KX301050
Ms1	Current study	I	1, 2, 3, 4, S1	KX301039
Ms2	Current study	I	1, 2, 3, 4, S1	KX301040
Ms3	Current study	I	1, 2, 3, 4, S1	KX301044
Cm1	Current study	I	1, 2, 3, 4, S1	KX301047
Cm2	Current study	I	1, 2, 3, 4, S1	KX301045

Cm3	Current study	I	1, 2, 3, 4, S1	KX301049
Cm4	Current study	I	1, 2, 3, 4, S1	KX301048
Si1	Current study	I	1, 2, 3, 4, S1	KX301056
Pa1	Current study	I	1, 2, 3, 4, S1	KX301060
Ge1	Current study	I	1, 2, 3, 4, S1	KX301061
Ch1	Current study	I	1, 2, 3, 4, S1	KX301038
Ki1	Current study	I	1, 2, 3, 4, S1	KX301059
Pta1	Current study	I	1, 2, 3, 4, S1	KX301058
Pta2	Current study	I	1, 2, 3, 4, S1	KX301057

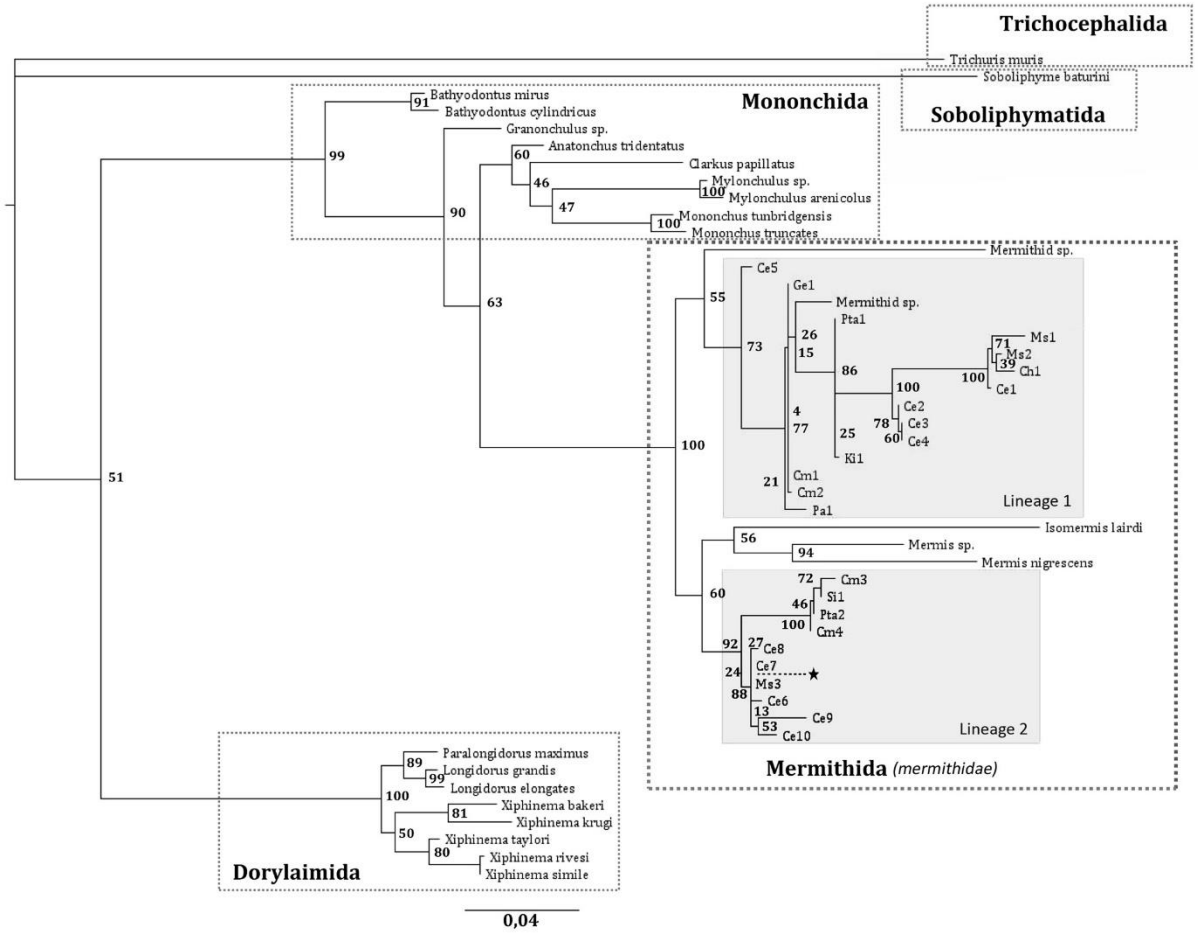
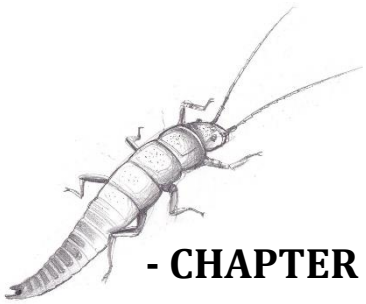


Figure S1. Maximum likelihood phylogeny of 48 Mermithid nematodes from Clade I. Among the 48 sequences, 24 are from the endoparasitic nematodes using *Timema* stick insects as hosts (sequences highlited in grey), and 24 are from previously published sequences (Ross *et al*., 2010). The different orders of Clade I are in bold and delineated by dotted lines. Bootstrap support was calculated using 1000 replicates. The black star indicates the position of the endoparasitic mermithid collected from a *Clitarchus sp* stick insect by Yeates and Buckley (2009).



- CHAPTER IV -

Evidence for the '*Parasite hypothesis for sex'* in *Timema* stick insects

C. Larose and T. Schwander

(Submitted in Evolution, in review)

Author Contributions: CL and TS conceived the project, conducted fieldwork, and performed the experiments. CL analyzed the data. CL and TS wrote the manuscript.

ABSTRACT

The *parasite hypothesis for sex* is one of the most frequently cited theories for explaining the maintenance of sexual reproduction in spite of the overwhelming costs generated by sex. This hypothesis suggests that sex allows organisms to respond effectively to continual changes in their environment, which is particularly relevant in the case of coevolution with parasites. Yet natural systems providing empirical support for this hypothesis are extremely scarce. We identified a fungal parasite that infects individuals from four independently evolved asexual *Timema* stick insect species and their sexual relatives throughout their distribution range. A combination of experiments allowed us to show that the parasite has strong negative fitness effects for the *Timema* hosts, induces an immune response, and that hosts are generally locally adapted to the fungal parasites. For three out of the four sexual-asexual species pairs, asexuals are more frequently infected by this fungal parasite than sexuals under natural conditions even if sexual populations are found in locations with higher parasite prevalence and virulence, providing strong support for the parasite hypothesis for sex. The pattern is reversed in the forth asexual-sexual species pair, highlighting the importance to consider lineage-specific effects in comparative studies of sexual and asexual species. Our study provides a rare test of predicted benefits of sex in a natural system.

Keywords: Sexual versus asexual reproduction, Parasite hypothesis for sex, Red Queen theory, Host-parasite interaction, Local adaptation, *Timema* stick insects, fungal parasites.

INTRODUCTION

Why sexual reproduction is maintained in the vast majority of lineages in spite of the significant costs it generates is a persistent question in evolutionary biology (Williams 1975; Maynard Smith 1978; West *et al.* 1999; Hamilton 2001; Kondrashov 2001; Otto & Lenormand 2002; Otto & Gerstein 2006; Otto 2009; Hartfield & Keightley 2012; Sharp & Otto 2016; Neiman *et al.* 2017). One of the most frequently cited theories for explaining the maintenance of sexual reproduction in natural populations is the "*Parasite hypothesis for sex"* (Levin & Levin 1975; Hamilton 1980; Bell 1982; Tooby 1982; Grosholz 1994; Howard & Lively 1994; Lively 1996; Otto & Nuismer 2004; Da Silva 2017). The main idea underlying this hypothesis is that coevolutionary interactions between parasites and hosts can drive continuously changing selection and thereby favor the maintenance of sex and outcrossing relative to asexuality (Jaenike 1978; Hamilton 1980). Assuming some form of genetic interaction between hosts and parasites which determines whether a parasite can successfully infect a given host, parasites are under selection to infect common host genotypes (Jaenike 1978; Hamilton *et al.* 1990). Sex can provide benefits in this situation because, contrary to asexual reproduction, it can generate offspring with rare, novel gene combinations, which would generally not be targeted by local parasites (Jaenike 1978; Hamilton 1980; Agrawal and Lively 2001).

There is a large body of research showing that parasites exert strong selection on their hosts, and that they often generate negative frequency dependent selection on host resistance genotypes (e.g., Dybdahl & Lively 1995; Little & Ebert 2001; Decaestecker *et al.* 2007; Duncan & Little 2007; Koskella & Lively 2009; Ashby & King 2015). However, although the *Parasite hypothesis for sex* has been much discussed, reviewed and refined

(e.g., Clarke 1976; Bell 1982; Seger & Hamilton 1988; see also review of Neiman & Koskella 2009), the empirical evidence that parasite-induced selection maintains sexual over asexual reproduction in natural populations remains scarce. The best evidence for parasites contributing to the maintenance of sex stems from mixed (sexual and asexual) populations of *Potamopyrgus antipodarum* snails and their endoparasitic trematodes (*Microphallus sp*). Different studies in this system showed that asexual snails prevail in areas associated with a low risk of infection (Lively 1987; Jokela & Lively 1995; Lively & Dybdahl 2000; Lively & Jokela 2002; Gibson *et al.* 2016) and that the parasites are generally locally adapted to the most common host genotypes (Dybdahl & Lively 1998). Long-term field data combined with laboratory experiments further showed that the frequency of different asexual clones fluctuated dramatically over time, in a pattern consistent with negative frequency dependent selection imposed by parasites, while the sexual snail populations remained much more stable (Jokela *et al.* 2009).

While there is strong evidence for parasites contributing to the maintenance of sex in *Potamopyrgus* snails, results from other natural systems are much more mixed, with little support for parasites contributing to the maintenance of sex overall (Neiman et al 2018). For example, Killick et al (2008) found no correlation between infection risk and the frequency of sex in the waterflea *Daphnia pulex.* In bagworm moth populations, parthenogenetic species are systematically less infected by parasitoids than their sympatric sexual counterparts, contrary to the predictions from the *Parasite hypothesis for sex* (Elzinga et al 2012). Finally, asexual geckos appear less susceptible to parasitism than their closest sexual relatives (Brown *et al.* 1995; Hanley *et al.* 1995). More empirical studies are therefore needed to evaluate how important parasite-induced selection is for the maintenance of sex in natural populations.

Here, we evaluate whether parasites generate selection for sexual reproduction in *Timema* walking stick insects. The *Timema* genus comprises several independently derived asexual lineages, each with a closely related sexual counterpart (Law & Crespi 2002a; Schwander *et al.* 2011). This allows for replicated comparisons between sexual and asexual lineages and therefore allows to disentangle reproductive mode effects from lineage-specific effects. *Timema* asexuals are also not polyploid or of hybrid origin (Schwander & Crespi 2009a), avoiding other confounding factors frequently present in sexual-asexual comparative studies.

We identified a widespread fungal parasite, most likely belonging to the genus *Aureobasidium*, infecting *Timema* individuals. We first verified that this fungal parasite triggers the host's immune system, negatively affects host fitness and is transmitted between *Timema* individuals. We then used a combination of experiments to test for local adaptation between *Timema* hosts and their fungal parasites. Finally, we quantified the frequency of the fungal infections in natural populations of sexual and asexual *Timema* species. Infection frequencies are difficult to interpret as they are jointly affected by local parasite prevalence and the level of host resistance to infection. To disentangle between the two effects, we performed transplant experiments in the field and examined if, as predicted by the *Parasite hypothesis of sex*, sexual individuals occurred in areas with higher parasite prevalence than asexual ones.

METHODS

Fungus identification

Fungal infections appear as black melanization marks mainly positioned at abdominal and thoracic cuticle segment intersections and at leg articulations of the insects. To identify the fungus causing these marks, we used a molecular approach based on sequence portions of the internal transcribed spacers ITS1 and ITS2 in six *Timema* individuals. For each individual we extracted DNA from a piece of cuticle containing a mark and from a similarly sized piece without a mark, following the DNA extraction protocol described by Miller et al. (1988). ITS1 and ITS2 were amplified using a nested-PCR as described by Martin and Rygiewicz (2005). Five µl PCR product were purified using 4µl of ExoI (20U/µl) (Thermo Scientific) mixed with FastAP Thermosensitive Alkaline Phosphatase (1U/µl) (Thermo Scientific). After addition of 5 µl of forward primer, purified PCR products were sent to GATC Biotech, Germany (www.gatc-biotech.com) for Sanger sequencing. Molecular identification of the fungus was then obtained by conducting BLASTN searches (BLAST v2.2.27+; Altschup et al. 1990) against the nr/nt database using Megablast (E value 1 e-4) (Zheng et al., 2000; Morgulis et al., 2008).

Effect of fungal infections on host immune response and fitness

To test if the fungal infections are detected by the host's immune system and have negative fitness consequences, we conducted experimental infections in the laboratory. Fungal spores for these infections were obtained by washing dried, field-collected plant material in deionized and autoclaved water. Plant material was collected from each *Timema* population used for experimental fungal infections (Table S1) such that fungal

spores would represent a heterogeneous mix of strains from all populations. Spores washed from the plants were plated on agar yeast extract medium in petri dishes, to allow for fungal growth and sporulation. Large amounts of spores were then harvested by washing dishes with 0.01% Triton X-100. The spore suspension was filtered to remove large pieces, and the spore concentration was determined with a hemocytometer and diluted to 10^7 spores ml^{-1}. For experimentally exposing *Timema* individuals to spores, we applied 5 µL of the spore suspension with a brush to the ventral side of their thorax. For the control treatment, we similarly applied 5 µL of 0.01% Triton X-100 (i.e., the suspension liquid without spores). Different infection experiments were then performed to study the immune response of *Timema* confronted with these infections, the appearance of marks on their body, and the potential effects on their fitness. Because it was not possible to obtain enough individuals from one species for all experiments, we used individuals from different *Timema* species and included species effects in analyses where appropriate.

To test whether *Timema* individuals mount an immune response following experimental exposure to fungal spores, we compared phenoloxidase activity of infected and control individuals 10 days after applying the treatments described above. Phenoloxidase is known to play a role in recognition and defense against fungal infections and is responsible for the activation of melanogenesis in invertebrates (Marmaras *et al.* 1996; Nappi & Christensen 2005; González-Santoyo & Córdoba-Aguilar 2012) . We used 30 males (15 treatment, 15 control) and 30 females (15 treatment, 15 control) of the sexual species *T. poppensis* for this experiment (with randomized assignment to treatments within each sex). For the individuals which survived the 10days, 10 µl of hemolymph was extracted with glass capillaries, added to 10µl PBS buffer and then stored at -80°C until

further use. To measure phenoloxidase activity, the hemolymph was thawed on ice and centrifuged at 5000g during five minutes at 4°C. Ten µL of supernatant was added to 90uL L-Dopa 3mM. The phenoloxidase activity was then measured every 15 sec at 490 nm during one hour at 30°C.

To test whether fungal infections had negative fitness consequences for the hosts, we compared survival and fecundity of control individuals with individuals experimentally exposed to fungal spores. For this experiment we used 85 females of three different species (60 *T. cristinae*, 19 *T. chumash* and 6 *T. shepardi)*. Half of the females of each species were used for the control treatment and the remaining half for experimental exposure to fungal spores as described above (with randomized assignment to treatments within each species). Daily survival was recorded for each female following the treatments and whenever a female died, we counted the number of black marks on the cuticle and the eggs laid during the experiment.

Tests of fungal transmissions between* Timema *individuals

In order to determine if the fungal infections are transmissible between *Timema* hosts, we monitored the survival of uninfected, focal females when placed in a cage with other ("resident") females that were either infected or not. Transmission experiments were conducted with three species, the two sexuals *T. cristinae* and *T. californicum*, and the asexual species *T. douglasi*, with individuals collected from the field and classified as "infected" if they had at least three black marks and as "uninfected" if they had none (sample sizes per species are summarized in Table 1). We set up 10 cylindrical plastic cages (30 cm high and 20 cm diameter) and added five to seven females labeled with a color dot on the ventral side (residents). For half the cages, the resident females were

infected, for the other half, resident females were uninfected (see Table 1). We then added eight or 10 focal uninfected females to each cage to test whether their mortality was higher if housed with infected resident females than with uninfected ones. For the two sexual species, we further added 6 to 7 uninfected males as we hypothesized they might mediate spore transmission between females in sexual species (males were not included in any analyses). The dead labeled females (infected or not) were left in the cages.

Table 1. Summary of the samples used for the fungal transmission experiment

Cage	Species[1]	Reproductive mode	Treatment	Number of focal females	Number and state of labeled females[2]
1	*T. californicum*	sexual	Control	**10**	6-uninfected
2	*T. californicum*	Sexual	Infection	**10**	6-infected
3	*T. cristinae*	Sexual	Control	**10**	5-uninfected
4	*T. cristinae*	Sexual	Infection	**10**	6-infected
5	*T. cristinae*	Sexual	Control	**10**	6-uninfected
6	*T. cristinae*	Sexual	Infection	**10**	5-infected
7	*T. douglasi*	Asexual	Control	**8**	6-uninfected
8	*T. douglasi*	Asexual	Infection	**8**	6-infected
9	*T. douglasi*	Asexual	Control	**8**	7-uninfected
10	*T. douglasi*	Asexual	Infection	**8**	7-infected

[1] All sampling locations are provided in Table S1

[2] In the cages 1 to 6, Half of the focal females were confronted to labeled females from their home location, and the other half were confronted to labeled females from a different location.

These experiments allowed us to compare the mortality between individuals which have been in contact with previously infected individuals, and individuals which have been in contact with healthy individuals. Furthermore, in the two sexual species, the 10 focal females came from two different populations (5 females per population) of which one was the same as the resident females (Table 1). This allowed us to investigate whether *Timema* have different susceptibilities to fungi from their own as compared to other populations.

Frequency of fungal infections in natural sexual and asexual populations

Timema stick insects were collected from 22 populations of eight different species throughout California in spring 2013 (Table. S1). An initial comparison between males and females in sexual species revealed no sex biased infection frequency or load (p-value = 0.39 for infection rate and p-value = 0.56 for infection load). We nevertheless only included females from sexual species for the most direct comparison between sexual and asexual populations. For each population, between five and 90 females (total 694; 262 sexual and 432 asexual ones; Table 1) were screened for fungal infections by counting the number of black marks present on the cuticle.

Several species of *Timema* feature a natural color polymorphism, with color morphs including different shades of green, yellow, beige, dark brown and dark grey (Sandoval 1994b). Previous studies showed that insects with melanized dark color morphs are better protected against infection by entomopathogenic fungi than insects with non-melanized, bright color morphs (Barnes & Siva-Jothy 2000; Dubovskiy *et al.* 2013a, b; Ortiz-urquiza & Keyhani 2013; Comeault *et al.* 2015). Among our sampled *Timema* populations, only populations of the species pair *T. cristinae/T. monikensis* comprised both bright and dark morphs. To avoid biasing infection rate estimates by morph frequencies in these two species, we selected populations with similar frequencies of bright and dark morphs, and we included only the bright ones in the analyses.

To compare infection rates and load between sexual and asexual species, we used a generalized linear model (GLM) as implemented in R (R Core Team 2017), with a binomial error distribution for infection rates and a poisson error distribution for infection loads. To account for the paired structure of sexual and asexual species in the *Timema*

phylogeny, we included a species pair effect in all analyses (with a reproductive mode by species pair interaction term).

Parasite prevalence in locations inhabited by sexuals and asexuals

Infection frequencies in natural populations are difficult to interpret as they are jointly affected by local parasite prevalence (infection risk) and the level of host resistance to infection. In order to estimate parasite prevalence in the populations surveyed for infection frequency, we performed transplant experiments in the field for four species pairs (*T. cristinae/T. monikensis*, *T. poppensis/T. douglasi, T. californicum/T. shepardi* and *T. podura/T. genevievae*). The aim of the transplant experiments was to estimate local parasite prevalence by introducing hosts from distant locations that had not co-evolved with local parasites. Thus, by measuring infection rates and loads of 'neutral' hosts, we can compare parasite prevalence between sexual and asexual populations without confounding effects of local host-parasite co-adaptation. We transplanted 'neutral' hosts to one population of *T. podura* and *T. genevievae*, and to two populations of all the other species allowing us to measure parasite prevalence in seven sexual and seven asexual populations. For each sexual population, we transplanted 20 sexual males and 20 sexual females, for asexual species, 40 females were transplanted. Transplanted individuals were placed in netbags on bushes of their original host plant species. A month later, we recorded mortality, and counted the black marks on the body of all surviving individuals.

RESULTS

Fungal infections

Molecular characterization of the fungus extracted from infected *Timema* individuals revealed similarities with three species of ubiquitous, yeast-like fungi of the genus *Aureobasidium* (*A. pullulans, A. proteae or A. microstictum*). The BLAST search indicated similarities from 96 to 99% between the *Timema* fungal parasites and each *Aureobasidium sp*ecies (e-values < 4e-126).

These *Aureobasidium* infections are detected by *Timema* individuals given they mount an immune response. The hemolymph of *T. poppensis* individuals experimentally exposed to fungal spores had a significantly higher phenoloxidase activity (Vmax mean = 0.71, SD = 0.30) than the hemolymph of control individuals (mean = 0.22, SD = 0.08; $F_{1, 18} = 16.9$, p < .0005; Fig .1A). Neither the sex of individuals or the interaction between sex and treatment significantly affected phenoloxidase activity (sex: $F_{1, 18} = 0.6$, p = 0.43; interaction: $F_{1, 18} = 0.2$, p = 0.63).

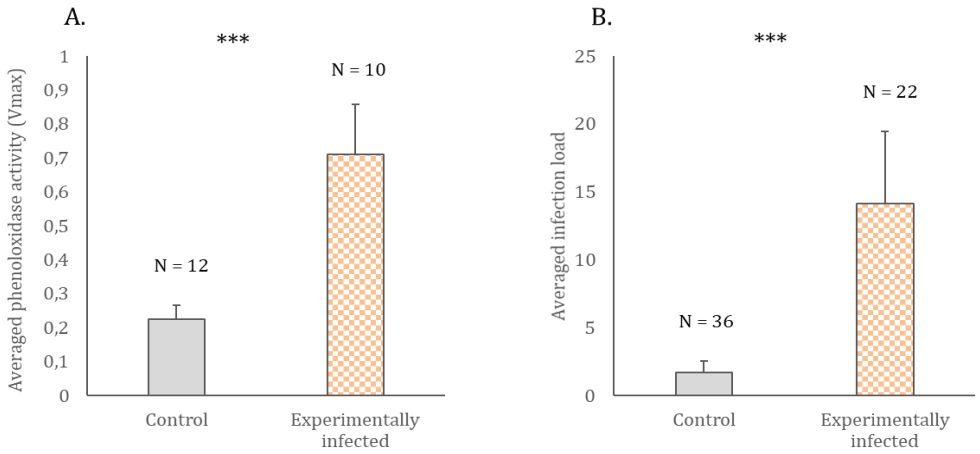


Figure.1. Phenoloxidase activity (A) and infection load (B) of control individuals and individuals experimentally exposed to fungal spores. Infection experiments were done with *T. poppensis*. Asterisks indicate significant differences between the control and the experimental treatment (p-value < 0.001).

Experimentally infected individuals of the species *T. cristinae, T. chumash, T. douglasi* and *T. shepardi* developed significantly more fungal marks (mean = 14.1 marks, SD = 10.6) than the control individuals (mean = 1.7 marks, SD = 1.7; $F_{1, 53} = 27.9$, $p < 3e\text{-}06$; Fig 1B). We also found that the species level had a significant effect ($F_{2, 53} = 14.6$, $p < 9e\text{-}06$; the species *T. douglasi* developed significantly fewer markers upon infection) while sex had no significant effect ($F_{1, 53} = 0.8$, $p = 0.377$). Monitoring the survival and fecundity of females experimentally exposed to fungal spores indicated that they survived significantly less (Kaplan-Meier test, $p < 4.1e\text{-}07$) and laid significantly fewer eggs per days (mean = 0.4 eggs/day, SD = 0.08) than control females (mean = 1.6 eggs/day; SD = 0.4; $F_{1, 58} = 159.5$, $p = 2e\text{-}16$). Indeed, following the infection treatment, individuals survived on average 22.4 days during which they laid about 10 eggs while the control individuals survived on average 37 days (Fig. 2) during which they laid about 57 eggs.

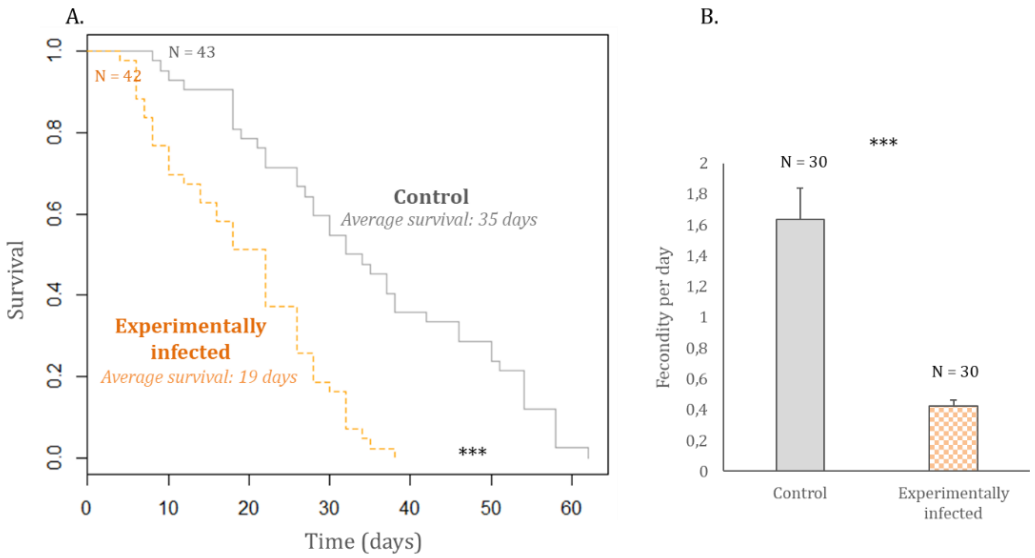


Figure 2. Survival (A) and fecundity (B) of experimentally infected and control individuals. Data based on a pool of 30 *T. cristinae*, 9 *T. chumash* and 3 *T. shepardi* females for each treatment. Fecundity is measured as the number of eggs laid per day until the death of individuals. Asterisks indicate significant differences between the control and the experimental treatment (p-value < 0.05).

Fungal transmissions between Timema *individuals*

Uninfected females of the species *T. cristinae, T. californicum*, and *T. douglasi* put in contact with naturally infected individuals died earlier than females which were in contact with uninfected individuals (Kaplan-Meier test, $p < 4.5e\text{-}07$; Fig. 3).

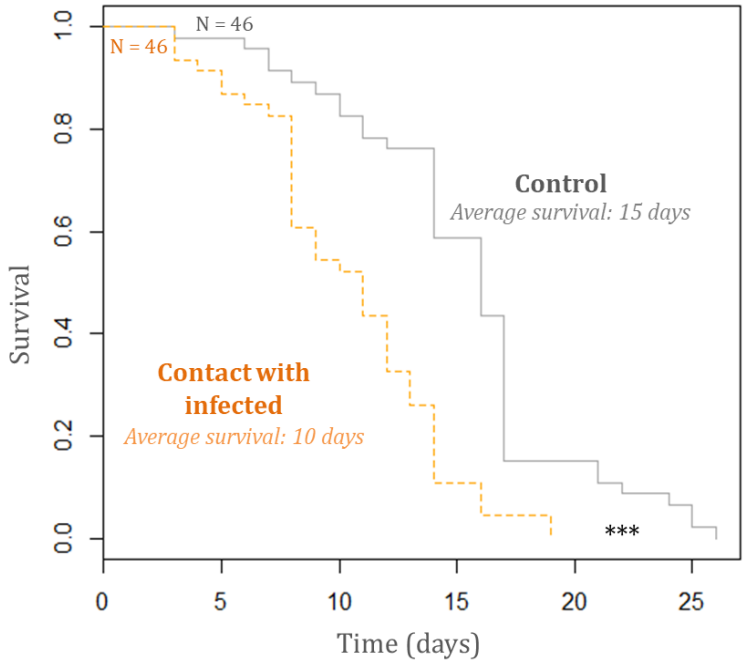


Figure 3. Survival of females in contact with infected and healthy individuals. The replicates per species are pooled.

Local adaptation between Timema *and the fungal parasites*

We found evidence indicating that *Timema* are locally adapted to fungal parasites, rather than the parasites being locally adapted to infect *Timema*. Controlled fungal transmission experiments in the laboratory revealed that females survived significantly less (mean = 8.2 days, SD = 4.18) when in contact with infected individuals from a different locality than when in contact with infected individuals from their own locality (mean = 12.53 days, SD = 3.6; Kaplan-Meier test, $p < 0.005$; Fig. 4).

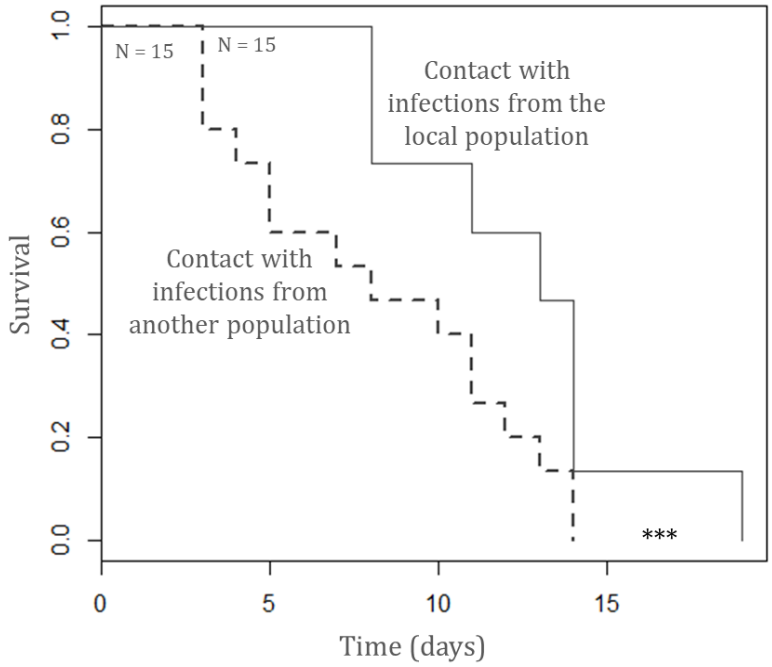


Figure 4. Evidence for local adaptation from the survival of individuals in contact with infected individuals from the local population or from another location. The full grey line represents the survival curve of the individuals in contact with the infected individuals from the local population, the dotted grey line represents the survival curve of the individuals in contact with infected individuals from another population than the local one. The replicates per species are pooled.

Fungal infections in natural populations

Infection rates and loads observed under natural conditions varied widely among the different studied *Timema* populations (rate: 0-71.8%, load: 1-19 marks, Table. S1). We modeled the infection rate and load as functions of the species pair and of the reproductive mode. We found an overall effect of "reproductive mode" on infection rate and load (infection rate: p-value < 0.007; load: p-value < 0.01) as well as interaction effects (infection rate: p-value = 0.068; load: p-value < 0.0002). In each species pair the asexual species had a higher infection rate than the sexual one, with the (marginally significant) interaction caused by a variation in the magnitude of the difference (Fig. 5A). For infection load however, the direction of the differences varied among species pairs, and we therefore analyzed each species pair separately. Fungal loads of infected females differed

significantly between sexuals and asexuals only in one species pair out of the three tested, with a higher load in the sexual species (*T. cristinae*/*T. monikensis*: p-value < 4.03e-05; *T. poppensis*/*T. douglasi*: p = 0.88, and *T. californicum*/*T. shepardi*: p = 0.11; Fig. 5B). Note that since not a single *T. podura* female was infected, we could not compare infection loads in the species pair *T. podura*/*T. genevievae* (Fig. 5B).

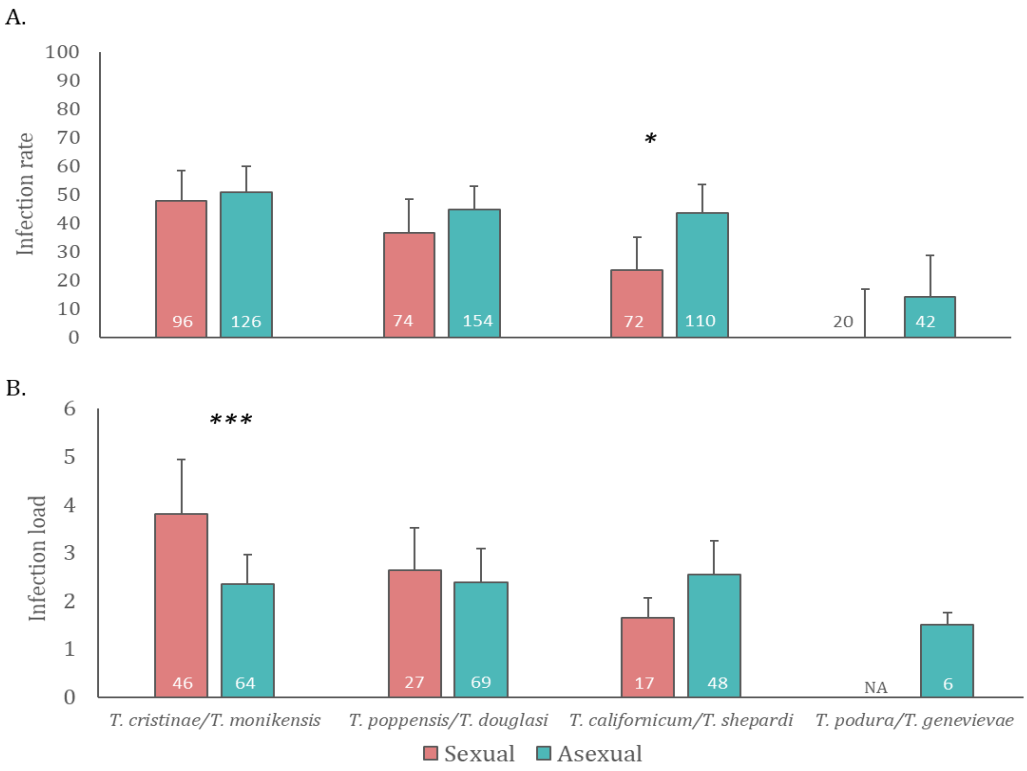


Figure 5. Infection rate (A) and load (number of fungal marks of infected individuals) (B) of sexual and asexual *Timema* species in natural populations. All populations per species are pooled for display. Sample sizes are indicated at the bottom of each bar. Infection load (B) is calculated from infected individuals and corresponds to the averaged number of fungal marks per infected individual of each species. *: p.value < 0.05; ***: p-value < 0.001

Parasite prevalence and virulence in locations inhabited by sexual and asexual populations

The higher infection rates of individuals in asexual than sexual populations could indicate higher parasite pressure in asexual than sexual populations. However, infection rates are

difficult to interpret as they are jointly affected by local parasite prevalence and the level of host resistance to infection. Given our finding above that *Timema* are adapted to resist local parasites, it is important to disentangle the two mechanisms. To do so, we compared parasite prevalence between sexual and asexual populations by measuring infection rates of individuals transplanted to different locations. Because transplanted individuals had no opportunity to co-evolve with local parasites, their infection rates are a better proxy for local parasites prevalence than infection rates inferred from local individuals. This approach revealed that sexual populations occur in locations with higher parasite prevalence (measured both via infection frequency and load) than asexual populations in three out of the four tested species pairs ($p < 0.05$ in the three pairs; Fig. 6A, B). In these three pairs, parasite virulence was also more pronounced in locations of the sexual than the asexual species, as survival was lower in locations of the sexual species ($p < 0.05$; Fig 6C). In the forth species pair (T. cristinae/T. monikensis), the pattern was reversed, as parasite prevalence was higher in locations inhabited by the asexual than sexual species ($p < 0.05$, Fig. 5A). The two species in this pair were further unusual as exposure to non-local parasites did not affect mortality rates, which were low (<10%) overall ($p > 0.12$; Fig. 6C).

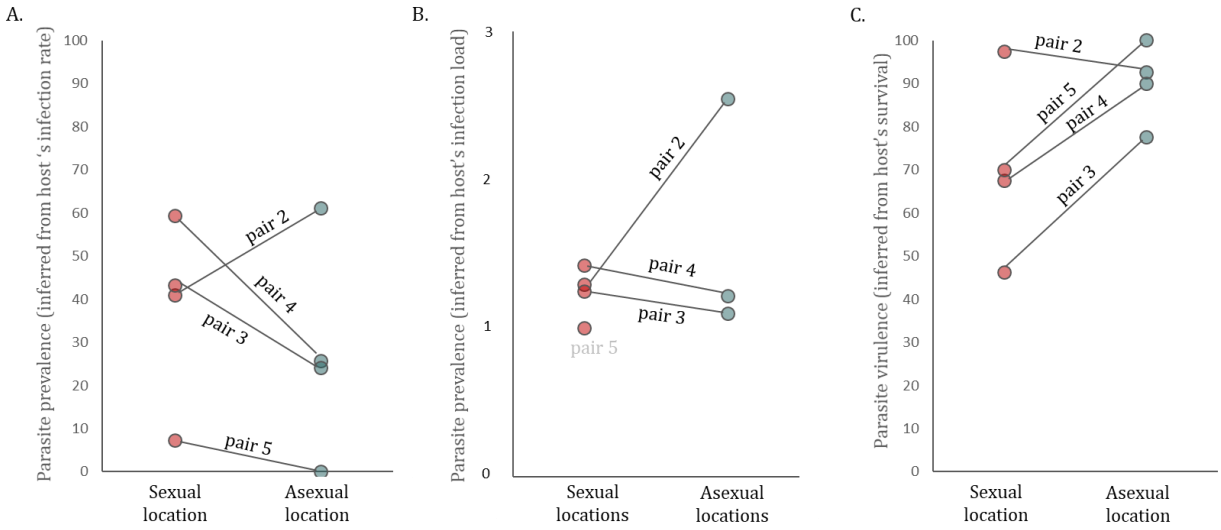


Figure 6. Parasite prevalence (A, B) and parasite virulence (C) in locations with sexual and asexual *Timema* populations. We inferred parasite prevalence in sexual and asexual locations from infection rates (A) and infection loads (B) of transplanted individuals, and parasite virulence (C) from mortality. Both locations within each species are pooled for display. Lines between two points connect sexuals and asexuals from each species pair. For panel (B), none of the individuals transplanted to the location of *T. genevievae* (pair 5) were infected, hence infection load cannot be estimated.

DISCUSSION

We isolated a fungal parasite infecting multiple species of *Timema* stick insects throughout their distribution range. This parasite is a mitosporic fungus closely related to the genus *Aureobasidium*, with a high degree of similarity to the known species *A. pullulans, A. proteae* and *A. microstictum*. These fungi are described as ubiquitous and are occurring in a wide range of habitats (Cooke 1959). *A. pullulans* was already described as parasitic but mainly of a variety of plant species (Cooke 1959; Herman *et al.* 1993; Woody *et al.* 2007; Cruywagen *et al.* 2015) although it has also been isolated from scale insect cuticles (Riesenberg 2000; Zacchi & Vaughan-Martini 2002). *Timema* are able to detect fungal infections, as they respond by mounting an immune response while melanizing the area of infection. The fungal infections further have a negative impact on the host's fitness and are transmissible between *Timema* hosts. These aspects are important prerequisites for potential co-evolutionary dynamics between the fungal parasite and *Timema* and provide the basis for testing the *parasite hypothesis for sex*.

The *parasite hypothesis for sex* predicts that sexual populations should be able to co-evolve with parasites more effectively than asexual populations, providing an advantage to sex. We found strong support for this prediction in three out of the four sexual-asexual species pairs of *Timema* that we tested. Indeed, in these three species pairs, although parasite prevalence and parasite virulence are higher in locations of the sexual species (Fig 6), the proportion of infected individuals is smaller in sexual than in asexual populations. This indicates that sexual individuals are better able to resist against their local parasites than asexual individuals while the asexuals are implanting and are maintained in areas where the parasite pressure is lower. In the forth species pair *(T.*

cristinae/T. monikensis), the parasite prevalence is higher in locations of the asexual species (Fig. 6) and the parasite does not seem virulent for the *Timema* host in any of the tested locations inhabited by *T. monikensis* and *T. cristinae*, providing no evidence for or against the *parasite hypothesis for sex*. Both sexuals and asexuals in this species pair thus seem to have developed a certain degree of tolerance towards these infections. Moreover, a possible explanation for why parasite prevalence is high in the studied locations of the asexual species *T. monikensis* is that *T. monikensis* is sympatric throughout its range with the sexual species *T. chumash* which we did not include in our surveys. Since the fungus infects all *Timema* species, local parasite communities are likely affected by the sexual as well as the asexual host.

Thus far the only strong evidence for the *parasite hypothesis for sex* in natural populations stemmed from *Potamopyrgus* mud snails and their trematode parasite (see Lively 1987, 2001; Dybdahl & Lively 1995; Jacobsen & Forbes 1997; Lively & Jokela 2002; Koskella & Lively 2007; Paczesniak *et al.* 2014; Vergara *et al.* 2015). Experimental evolution studies also show that sex and outcrossing can provide benefits under strong parasite pressure (e.g., Auld *et al.* 2016), but results from such studies are generally difficult to extrapolate to natural conditions (Meirmans *et al.* 2012). This lack of broad empirical support from natural systems is surprising given the widely held belief that parasites provide the most likely explanation for why sex is so overwhelmingly successful. Our experiments in *Timema* offer important empirical support for the idea that parasites indeed generally contribute to the maintenance of sex.

Surprisingly, we found that *Timema* hosts are locally adapted to fungal parasites, rather than local adaptation of the fungal parasites to the *Timema* hosts. Under laboratory

conditions, individuals featured lower mortality when in contact with infected individuals from their own population than when in contact with infected individuals from other populations. Local adaptation of the host is surprising as parasites are typically more strongly locally adapted than their hosts. Indeed a number of transplantation experiments between hosts and their parasites in other systems found strong local adaptation of parasites to the hosts (Ballabeni & Ward 1993; Koskella *et al.* 2000; Lively *et al.* 2004; Greischar & Koskella 2007; Laine 2008). This pattern is believed to stem from the fact that parasites typically have larger population sizes, shorter generation times and higher rates of mutation and migration than their hosts, allowing for faster rates of adaptive evolution (Price 1980). Nevertheless, there are a number of factors that can prevent parasites from becoming locally adapted, and instead induce maladaptation of the parasite or facilitate local adaptation of the host to the parasites as it is the case in the *Timema*-fungi system (Gandon *et al.* 1996, 1998; Gandon 2002; Gandon & Michalakis 2002). The perhaps most likely explanation for the *Timema*-fungi system is that *Timema* hosts and the fungal parasites differ in their degree of specificity. Indeed, *Timema* stick insects, because of the dramatic effect of the fungal infections on their fitness and the strong immune response against experimental infections in the lab are adapted to defend specifically against parasitic fungi while these fungi may be less specific in their host targets, infecting other insect species co-occurring with *Timema*. Theoretical studies have shown that if fitness effects on hosts are considerable and parasites use multiple different hosts, trade-offs among adaptations to different hosts can result in local adaptation of the host rather than the parasite (see Gandon 2002).

To conclude, our results provide strong support for parasites contributing to the maintenance of sex (i.e., *the parasite hypothesis for sex*) in three of four sexual-asexual

Timema species pairs. Our study is to date the first to investigate this theory by making several replicated comparisons between sexual and asexual lineages and shows that it is essential to replicate tests at the lineage level. The reasons for why in one species pair, parasite prevalence is higher for locations with populations of the asexual species remain to be investigated. Moreover, determining whether parasite pressure alone is sufficient to compensate for the costs of sex in *Timema*, or whether pluralist mechanisms are required (West *et al.* 1999; Neiman *et al.* 2017), remains a challenge for future studies.

Acknowledgments

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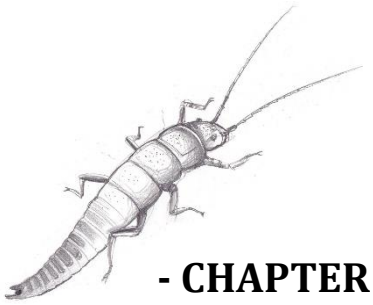
SUPPORTING INFORMATION

Table S1. Sampling locations of individuals from the different *Timema* population used in this study

Species	Reproductive mode	Populations[1]	GPS coordinates	Number of individuals[2]
T. cristinae	Sexual	Ojai1 *	34°30'20.0"N 119°16'47.5"W	32
		Ojai2 * •	34°31'59.6"N 119°14'47.5"W	0
		WTA* ¤ •	34°30'22.3"N 119°46'05.3"W	64
T. monikensis	Asexual	For Sale •	34°06'53.6"N 118°51'11.3"W	90
		Sycamore •	34°06'33.7"N 118°54'51.0"W	36
T. poppensis	Sexual	Bear Creek ¤	37°09'56.2"N 122°00'56.4"W	40
		Big Pullout Vista	37°24'39.9"N 122°18'21.4"W	5
		Iverson	38°37'05.9"N 123°17'29.8"W	16
		Madonna ¤	37°01'07.5"N 121°43'32.0"W	7
		Swanton	37°05'11.1"N 122°15'08.8"W	6
		Fish rock •	38°49'05.1"N 123°35'03.5"W	0
		Yerba •	37°27'24.5"N 122°20'19.8"W	0
T. douglasi	Asexual	Fort Bragg	39°31'08.4"N 123°29'52.8"W	41
		Sherwood	39°32'16.8"N 123°27'03.6"W	8
		Manchester ¤ •	38°58'57.2"N 123°28'10.4"W	69
		Orr Springs1 * ¤ •	39°12'44.5"N 123°18'30.2"W	36
T. californicum	Sexual	Hamilton	37°20'30.5"N 121°38'34.8"W	24
		Saratoga * •	37°11'47.0"N 122°02'27.1"W	48
		Summit *	37°02'43.2"N 121°45'11.6"W	0
		Fremont •	36°45'48.7"N 121°30'09.1"W	0
T. shepardi	Asexual	Willits	39°25'32.8"N 123°17'49.2"W	6
		Manchester	38°57'22.4"N 123°32'04.9"W	14
		Elk ¤ •	39°16'42.2"N 122°55'39.6"W	60
		Orr Springs2 •	39°12'02.2"N 123°17'38.1"W	30
T. podura	Sexual	EDNA	33°53'06.7"N 116°51'35.2"W	20
		HW74 •	33°39'26.9"N 117°23'50.9"W	0
T. genevievae	Asexual	Antonio •	37°19'42.0"N 121°29'07.6"W	35
		HW20	38°59'38.4"N 122°31'26.4"W	7
T. chumash	Sexual	HW2 ¤	34°15'42.4"N 118°06'27.6"W	0

[1] All populations used in this study. The symbol * indicates the populations used for the experiment testing for fungal transmissions between *Timema* individuals performed in the lab. The symbol ¤ indicates the populations used for the experimental infections performed in the lab. The symbol • indicates the populations used for the transplant experiment in the field. The cells highlighted in gray correspond to the populations used to quantify the fungal infections in natural populations

[2] corresponds to the number of individuals used to quantify the fungal infections in natural populations



- CHAPTER V -

Testing for reproductive polymorphism in the *Timema poppensis - T. douglasi* complex

C. Larose, D. J. Parker, K. Légeret, T. Schwander

(in preparation)

Author Contributions: CL conceived the project. CL and DP conducted fieldwork. CL, DP and KL performed the experiments. CL analysed the data. CL wrote the first draft of the manuscript. TS provided input and revisions.

ABSTRACT

The *Timema* genus has become one of the major group of organisms for the study of many evolutionary issues related to the mysterious predominance and maintenance of sex despite its theoritical costs compare to asexuality. Indeed, seven independently derived obligate asexual lineages from five species have been identified in this group, each with a closely related sexual counterpart. To date, no mixing between sexual and asexual individuals from a given species pair was observed in this group. It was therefore impossible to study the direct costs and benefits of these two reproductive strategies in the short term while competing under natural conditions. In the present study, we report and examine new *Timema* populations previously considered as part of the obligate parthenogens *T. douglasi*. Both populations aroused our interest when unusual proportions of males were found among the females. In order to precisely characterize these populations, we performed two transects recording precise sex-ratios along both corridors. In the first transect we found 50:50 sex-ratio locations followed at only a few meters distance by 100:0 sex-ratio locations. In the second transect, we found that some 100% female spots alternated with some spots containing 9 to 23% males. For each sampling location from both transects, we then studied the hatching timing, hatching success of both virgin and mated females as well as the mating behavior of virgin females. Our preliminary results indicate an extremely high reproductive polymorphism present in these populations, with, for the first time in the *Timema* genus, the existence of mixed populations where obligate sexuals and asexuals co-occur, as well as the existence of facultative parthenogens. Interestingly, this study questions the status of "species" in *Timema*, and specifically the status of the *T. poppensis* (obligate sexual) / *T. douglasi* (obligate asexual) species pair, which appears to be rather a species-complex with an extreme reproductive plasticity.

Keywords: Hatching success, Mating behavior, Reproductive polymorphism, Sexual versus asexual reproduction, Sympatry versus allopatry, Sex-ratio, *Timema* stick insects.

INTRODUCTION

Even though the vast majority of organisms are reproducing using sexual reproduction, there are many other ways to reproduce, including different forms of asexual reproduction. Different reproductive strategies have different consequences on the potential of diversification and adaptation of populations and ultimately influence the evolution and structuration of biodiversity (Maynard Smith 1978). However, despite its essential role, our understanding of the evolution of reproduction is still incomplete. In particular, explaining the widespread occurrence of sexual reproduction throughout the animal and plant kingdoms, despite the potential advantages of asexual reproduction, is one of the greatest challenges for evolutionary biology (Maynard Smith 1978). This enigma has generated a large body of theories aiming to explain the rarity of parthenogens among multicellular taxa (e.g., Hill & Robertson 1966; Bell 1982; Kondrashov 1988; Barton & Charlesworth 1998; West *et al.* 1999; De Visser & Elena 2007; Otto 2009). However, to date all the theories are rarely supported by empirical evidence (Neiman *et al.* 2018). It is therefore essential to evaluate precisely the costs and benefits of these two modes of reproduction in nature both in the short and in the long term.

An ideal biological system for attempting to precisely quantify costs and benefits of sexual and asexual reproduction would be a system in which there are sexual and asexual populations living in sympatry (i.e., living in the same geographic area), and sexual and asexual populations living in allopatry (i.e., occurring in separate, non-overlapping geographic areas). Indeed, such a system would allow us to compare environments inhabited by sexual populations and environments inhabited by asexual populations and thus to highlight the ecological conditions favoring each reproductive strategy over the

mid and long term. In addition, areas of sympatry would allow us to disentangle the direct costs and benefits of these two reproductive systems in situation of competition and thus in the short-term. To date however, very few such sexual-asexual biological systems with these criteria are known, and the rare ones differ in important attributes as polyploidy, or are from hybrid origin (see Schön *et al.* 2009).

In this study, we report and describe new populations belonging to the genus *Timema*, which potentially correspond to unique cases of sympatry between sexuals and asexuals in this genus. *Timema* is a small genus consisting of 23 known species of stick insects (Phasmatodea: Timematidae) that comprise at least five obligate parthenogens and 16 obligate sexual species (Sandoval *et al.* 1998; Vickery & Sandoval 1999, 2001; Schwander & Crespi 2009a). Species of this genus are native to the western USA and Mexico. Seven independently derived asexual lineages from five species have been identified in this group, each with a closely related sexual counterpart (Law & Crespi 2002a; Schwander *et al.* 2011). To date, all the described sexual and asexual *Timema* species from a given species pair live in allopatry. In addition, the five asexual species are described as obligate parthenogens, which means that fertilization of oocytes does not occur even when mated with males of the sexual-sister species in the lab (Schwander *et al.* 2013).

Regarding the sexual species, a previous study from Schwander and Crespi (2009) investigated the extent of spontaneous parthenogenesis (i.e., tychoparthenogenesis) among females from nine *Timema* sexual species. This study found that 30.4% of the virgin sexual females tested (n=204) produced unfertilized eggs that gave rise to some viable offspring. The fitness of the offspring produced spontaneously by sexual females in absence of fertilization compared to offspring produced by obligate parthenogens is

unknown. However, despite the apparent ability of virgin sexual females to spontaneously produce offspring via automictic parthenogenesis, the known and described sexual populations of *Timema* are characterized by balanced sex-ratios close to 50:50 (Schwander *et al.* 2010). On the contrary, the asexual populations are characterized by populations of females close to 100:0 sex-ratio. Rare males are sometimes found (<1%) within asexual population, which are likely produced by aneuploidy. Indeed, since sex determination in *Timema* is an XX (female): XO (male) system (Schwander & Crespi 2009a; Schwander *et al.* 2013), the loss of an X chromosome during reproduction will result in the production of a male.

In *Timema*, description of new species was based firstly on morphological criteria, and secondly on sex ratios recorded in natural population. Each parthenogenetic species has a sexual relative species containing morphologically identical females. Sex ratios were then used to characterize the different "species" within each sexual-asexual species pair. A population consisting exclusively of females was considered to be part of a parthenogenetic species and a population containing males was considered as part of a sexual species. In the case of the *T. poppensis/T. douglasi* species pair, Sandoval and Vickery first discovered and described the parthenogenetic species *T. douglasi* constituted only by females in 1996, and they only found a few years later, in 1999, a population containing morphologically similar females living with males, resulting in the description of the *T. poppensis* sexual species.

In the present study, we specifically examine in detail two natural populations currently considered as part of the obligate asexual species *T. douglasi*. The two populations have been routinely sampled along two roads in California for several kilometers (Fig. 1) across

multiple field seasons. Unexpectedly, such sampling found that the incidence of asexual males in these populations was higher than we would expect from aneuploidy, sometimes exceeding 23%. The precise geographic distribution of both *T. poppensis* and *T. douglasi* is currently unknown. In order to precisely characterize these populations and the mode of reproduction of the females constituting them, we performed two detailed transects to record the precise sex-ratios across both locations. In the first one (called "Orr transect") we found 100% female spots alternated with spots containing 9 to 23% of males suggesting either i) mixed populations of sexual and asexual individuals co-occurring, ii) the existence of facultative parthenogens or iii) an unusually high production of accidental males by obligate asexual females in some locations only. In the second transect (called "Manchester transect") we found about 50:50 sex-ratio locations followed, at only few meters distance, by 100:0 sex-ratio locations suggesting an overlap or a very close proximity between a sexual and an asexual population. In order to understand precisely the composition of these populations, we then studied the hatching timing and hatching success of both virgin and mated females, as well as the mating behaviors of females present in each of the sampling locations.

This study allows us to clarify the reproductive mode of unusual *Timema* populations, and ultimately to question the status of "species" in this genus. The “species pair” *T. poppensis*/*T. douglasi* seems to be rather a species-complex with an extreme reproductive polymorphism, ranging from obligate parthenogens to obligate sexuals, with the presence, for the first time in the *Timema* genus, of facultative parthenogens.

METHODS

Sex-ratios in natural populations

In order to characterize the reproductive system of the *Timema* stick insects living in areas of potential overlap between *T. poppensis* and *T. douglasi*, we first characterized the sex-ratios of 29 sampling locations constituting them. We performed two transects; respectively referred to as "Manchester transect" and "Orr transect" (Fig. 1). For both transects, we chose the sampling locations according to the host plant distribution along the main road, with separate patches of douglas fir (*Pseudotsuga menziesii*) trees considered as distinct sampling locations. Using sweep nets, we sampled all *Timema* from a given tree patch for 2 hours with approximatively constant sampling intensity, such that the number of *Timema* collected can be used as a proxy for the population density at each location. We collected between 3 and 270 individuals (all juvenile) per sampling location for a total of 1195 individuals for the Manchester transect (864 females and 331 males) and 1074 individuals (1029 females and 45 males) for the Orr transect (2269 insects in total).

In order to detect potential sex ratios' fluctuations over time, we recorded the sex ratio of a sub-set of locations across multiple years. Specifically, we estimated the sex ratio of *Timema* in eight sampling locations in three different years in May (i.e., 2014, 2015 and 2017), sex-ratios for the remaining 21 sampling locations were only estimated once in 2017.

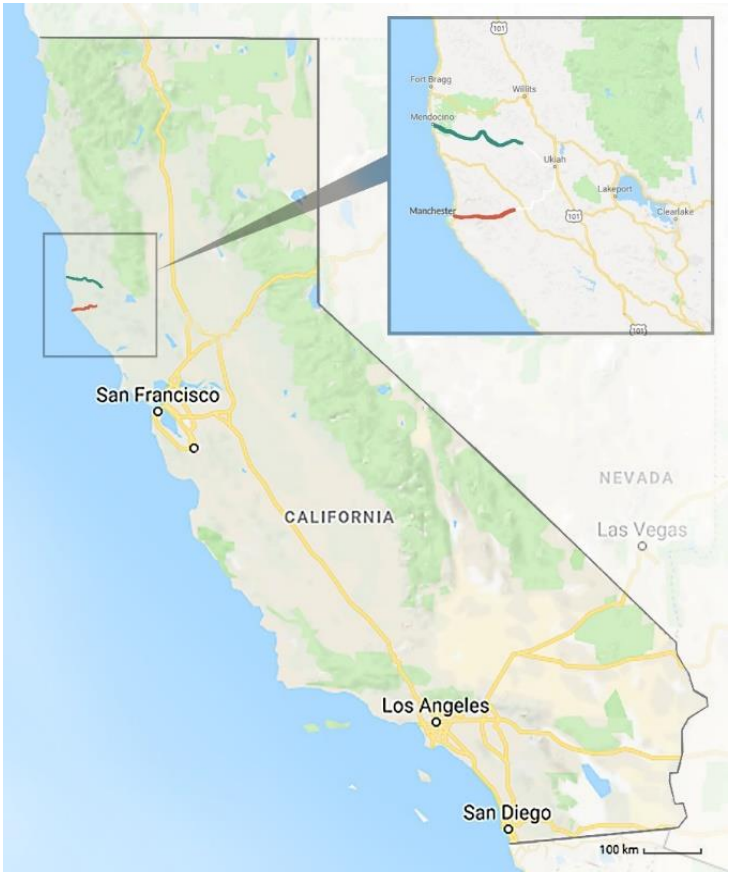


Figure 1. Locations of the studies transects. The red line corresponds to the Manchester transect and the blue line to the Orr transect.

Hatching timing and success of eggs laid by virgin and mated females

We aimed to obtain hatching successes of unfertilized and fertilized eggs for as many females as possible. For each sampling location, individuals were separated by sex following collection and then maintained in square cages of 30 x 30 cm on douglas fir host plant. Since all individuals were juvenile upon collection, this allowed us to obtain virgin females for all locations (only adult individuals are able to mate). All cages were kept in a climatic chamber at 23°C, 55% humidity and 12:12h day:night cycle. Once individuals had reached adulthood we individually isolated all females in Petri dishes to obtain unfertilized eggs. Fresh food (a small branch of douglas fir) and a moistened piece of cotton wool were added every two days in each Petri dish. These virgin females (309 females in total) were left to lay unfertilized eggs.

From these 309 virgin females, 165 females were randomly selected to record mating behavior (see below). For the remaining 144 females we waited for them to lay a minimum of 20 unfertilized eggs. We then allowed females to mate with a male from sampling location with about 50:50 sex-ratio. To qualify a female as "mated", copulation was visually confirmed. Some females died before they had laid 20 eggs and therefore before they could be mated. As a consequence, we only had one set of unfertilized eggs for these females. In addition, some females used in the mating behavior experiment mated with a male before having laid a single unfertilized egg. We thus only had one set of eggs for theses females as well, i.e., eggs laid after mating (see Table. 1). All eggs laid by each female (both the ones used in the mating behavior experiment and the ones we kept and observed separately), were collected and counted until the female's death. For each female, the unfertilized eggs and the eggs laid after mating were kept separately until hatching, which occurs after approximately 5-6 months of diapause. The eggs began to hatch on October, 27th of the same year. The eggs were then checked every other day and the number of hatchings recorded. No eggs hatched after December 27th. We only included females that laid a minimum of 5 eggs for further analysis (Table. 1)

For a subset of females (94 in total), we obtained enough eggs before and after mating for comparison of the hatching successes. To identify different types of females based on their sexual and asexual reproductive abilities, we performed a hierarchical clustering analysis. For example, we hypothesized that if there were obligately sexual females, these females would all be represented by low hatching success of unfertilized eggs and high hatching success of fertilized eggs. By contrast, the obligate asexuals would have a high hatching success of unfertilized eggs, the mating having presumably no effect on their fertility.

Females for which we only had one set of eggs (laid before or after mating) were used to study, from a larger amount of data, the averaged fertility of the females present in each sampling location. To this aim, we estimated the hatching timing, and the hatching success of all the virgin (309 females in total) and all the mated females (185 females in total) of this study. In addition, we compared the hatching success frequencies distributions of all virgin females and of all mated females observed in the different locations using Kolmogorov-Smirnov Tests (Smirnov 1939) implemented in R (R Core Team 2017). Finally, we studied whether the pre- and post-mating hatching successes were correlated to the sex ratios of the sampling locations.

Table 1. Number of virgin and mated females from each sampling locations of the transects used for the study of egg hatching success and timing[1]

Manchester transect	**M1**	**M2**	**M3**	**M4**	**M5**	**M6**	**M7**	**M8**	**M9**	**M10**	**M11**	**M12**
virgin	15	27	13	37	1	20	5	3	33	15	1	25
mated[2]	2	8	1	28	0	27	3	0	21	25	0	30
virgin and mated[2]	8	0	1	8	13	2	3	0	9	9	0	11
Orr transect	**O1**	**O2**	**O3**	**O4**	**O5**	**O6**	**O7**	**O8**	**O9**	**O10**	**O11**	**O17**
virgin	15	1	6	40	0	0	16	22	0	11	3	0
mated[2]	14	1	2	10	0	0	9	20	0	1	1	3
virgin and mated[2]	5	1	2	7	0	0	3	10	0	1	1	0

[1]only females which laid a minimum of five eggs
[2]only females with confirmed copulation are included.
Grey cells correspond to sampling locations in which we found males.

Mating behavior

In order to investigate the mating behavior of the individuals inhabiting the locations of this study, we randomly picked 165 virgin females and 165 virgin males from nine

locations of the Manchester transect (M1, M2, M3, M4, M5, M6, M9, M10, M12) and from six locations of the Orr transect (O1, O4, O5, O7, O8, O17, see Fig. 3) in which we recorded different sex-ratios. We first installed individually each of the 165 females in a Petri dish, randomly distributed on a table, independently of the population of origin. Males were then randomly picked and added one by one in each Petri dish. This allowed us to form 165 couples. As soon as the two individuals of a given couple were together in the dish, we started to record the time for this couple (Fig. 2A). From this moment, two observers scanned and recorded constantly throughout the experiment different behaviors occurring between each individual constituting these couples for 12 hours. We recorded the time i) of the first contact between the two individuals, ii) when the male started to guard the female (Fig. 2B), iii) when they started to mate (Fig. 2C), iv) when the mating stopped, v) when the male stopped to guard the female.

Figure 2. Illustrations of *Timema* couples during the mating behavior experiment: (A) A male (on the top right of the picture) is introduced in a Petri dish containing a female (at the bottom left of the picture); (B) A guarding behavior with a male sitting on a female; (C) A mating event between a male (on the top) and a female.

We finally studied whether these different mating behaviors carried out by virgin individuals from different sampling locations were correlated to the sex ratio recorded in these locations, and thus were influenced by the presence of males in their natural habitat.

RESULTS

Sex-ratios in natural populations

Along the "Manchester transect", we sampled 12 localities. This allowed us to highlight a very close proximity between locations with an approximately 50:50 sex-ratio (varying between 43:57 and 55:45) and locations with a sex-ratio of 100:0 (Fig. 3A). This suggests, for the first time in the *Timema* genus, a very close proximity or even an overlap between a sexual population and an asexual population. For the "Orr transect", we sampled 17 localities and found locations with a 100:0 sex-ratio, which alternated with locations containing 9 to 23% of males (Fig 3B).

Comparing sex ratios of three different years for eight localities revealed that populations with female-biased sex ratios are always strongly female-biased (Fig. 4). It is however interesting to note that among these female-biased locations, the three locations which contained a small fraction of males (i.e., 9-23%), contained males each year of collection, while the four locations which contained 100% of females in a given year, contained 100% of females without a single male each year of collection (Fig. 4). By contrast, the only sampling location with a 2017 sex-ratio close to 50:50 and multiple sampling years featured important sex-ratio fluctuations including a very male-biased sex ratio in 2014 (i.e., 29:71; Fig. 4).

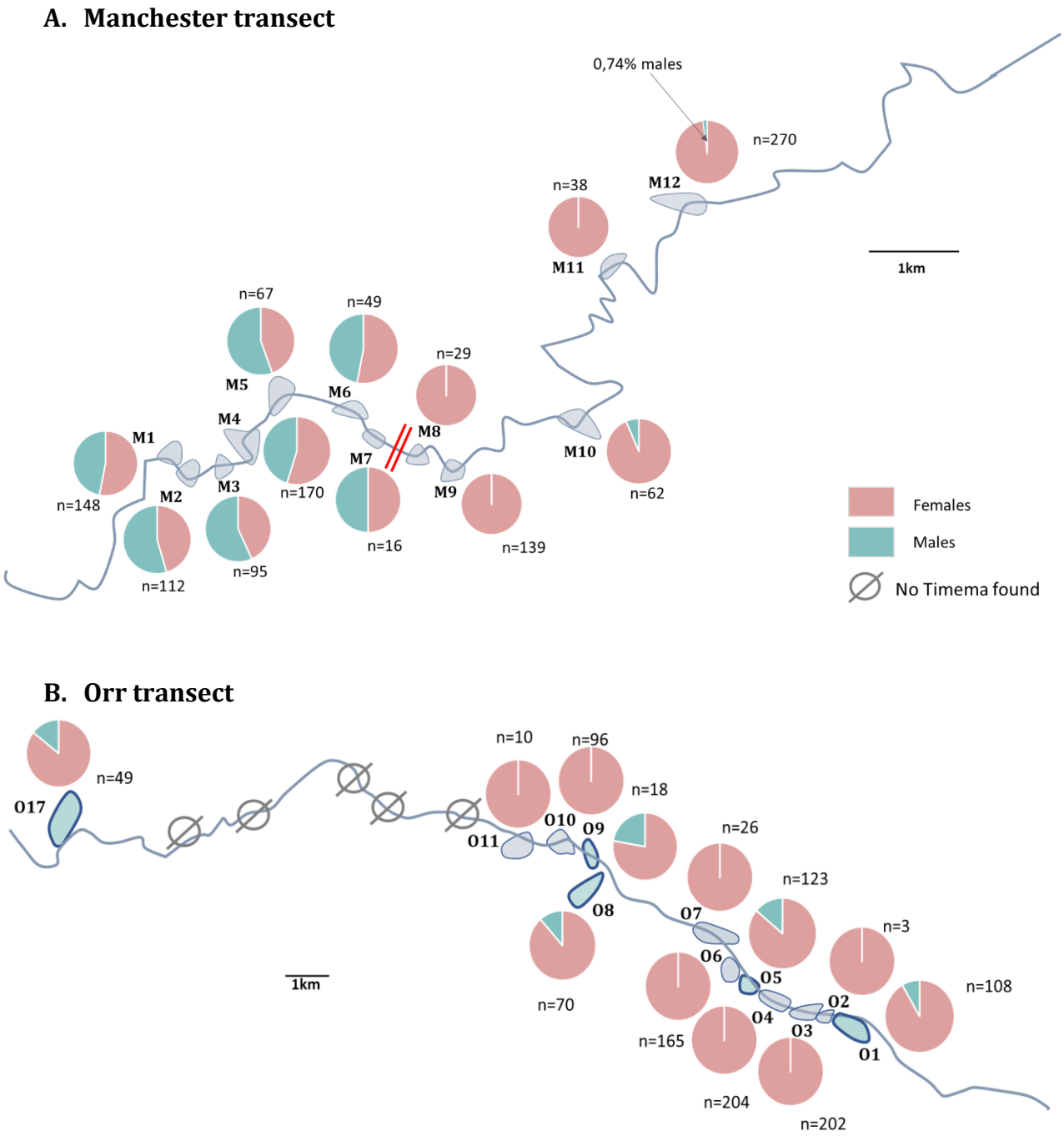


Figure 3. Sex-ratio record along two transects. In each circle, the respective proportion of males and females are indicated in blue and pink. Red lines within the Manchester transect symbolize the boundary between the zone containing a sex-ratio close to 50:50 and the zone containing a 100:0 sex-ratio.

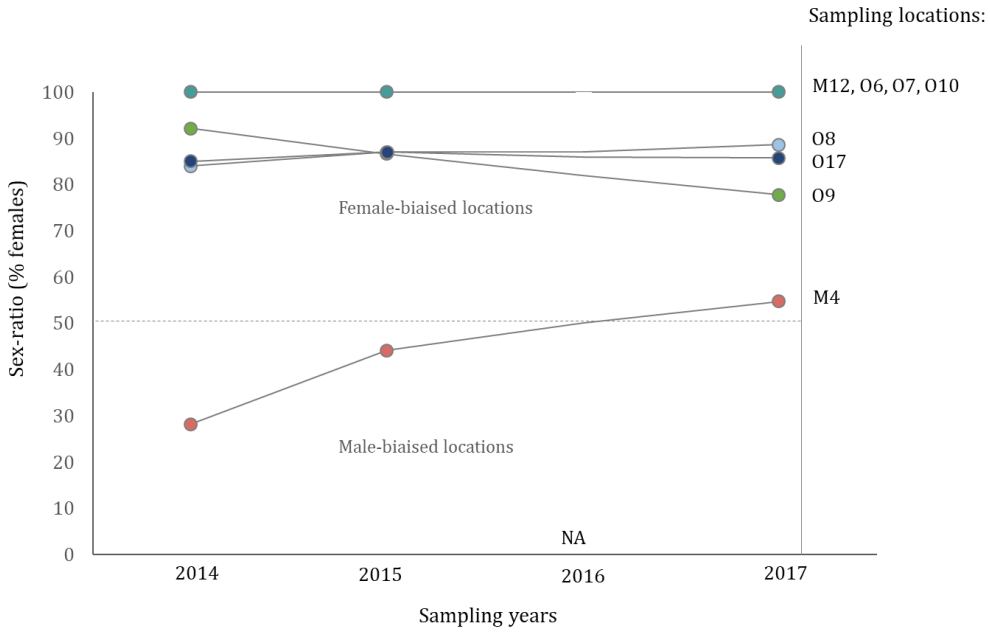


Figure 4. Sex-ratios recorded during successive years.

In order to characterize the reproductive mode of the individuals from different locations, we studied the reproductive timing and success of virgin and mated females from each sampling location (Table.1)

Hatching timing and success of eggs laid by virgin and mated females

Our analyses of egg hatching success of virgin and mated females revealed high inter-individual variability of sexual and asexual reproductive abilities. Across all locations, there appeared to be a continuum of females with very variable capacity to reproduce asexually (ranging from 0 to 100% with the full range of possible intermediaries; Fig.5; Fig. S1). A cluster analysis nevertheless distinguished three broad categories of females: i) females with poor reproductive success (<50% hatching success) both pre and post mating, that we called "*poor reproducers*" (Fig. 5; blue dots) ii) virgin females with a poor reproductive success (<50% hatching success) but a significantly higher hatching success post mating (varying between 40 and 100%), that we called "*efficient sexual reproducers*"

(Fig. 5; yellow dots), and iii) virgin females having a good hatching success (varying between 61 and 100%), that we called "*efficient asexual reproducers*" (Fig. 5; red dots).

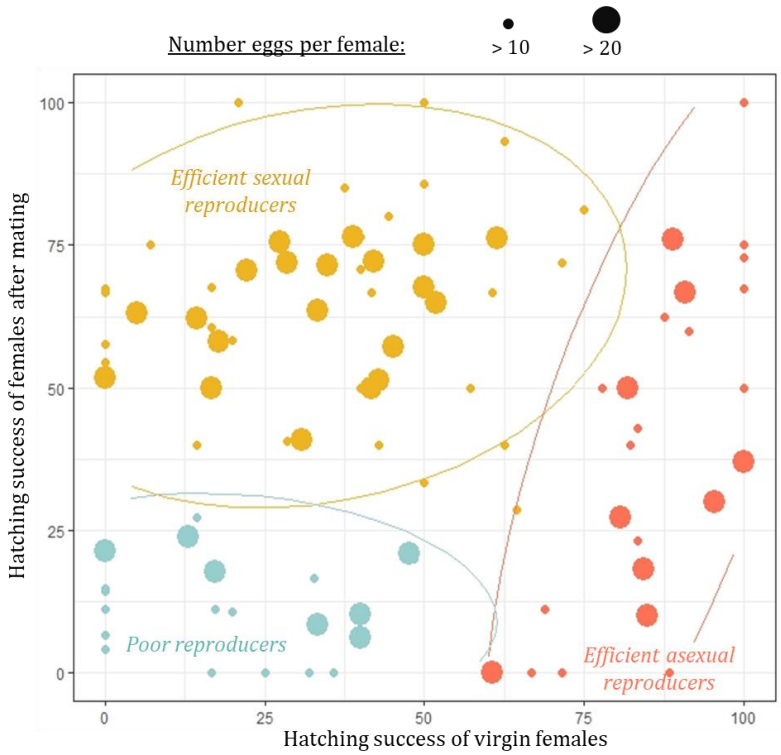


Figure 5. Variable sexual and asexual reproductive abilities characterize the females of this study. Shown is the hatching success of different females before and after mating. Each dot corresponds to a single female. A hierarchical cluster analysis was performed to discriminate the different groups present in the scatterplot (different colors represent different groups).

Females belonging to the "*poor reproducers*" category occurred in several sampling locations from both transects, without any apparent geographical pattern (Fig. S2.A). Females of the "*efficient sexual reproducers*" category occurred mostly in locations with a high proportion of males (r=0.81, p-value < 6.1e-5; Fig. S2.B), while females of the "*efficient parthenogens*" category occurred mostly in populations without males (r=-0.66, p-value<0.01; Fig. S2.C).

Previous studies have shown that in many facultatively parthenogenetic species, unfertilized and fertilized eggs did not have the same developmental time until hatching, with the unfertilized eggs developing more slowly (e.g., Funk *et al.* 2010; Liegeois *et al. in prep*), and hatching spread out over a longer period of time (e.g., Humpesch 1980; Harker 1997; Matsuura *et al.* 2004; Matsuura & Kobayashi 2007). In *Timema*, the average developmental time of eggs laid by virgin females was not distinct from the developmental time of eggs laid by mated females in any of the population (data not shown). In addition, hatching spread out over exactly 55 days in both cases.

Analyses of the fertility of a larger number of females including the 94 females for which we had both eggs laid while virgin and eggs laid after mating, and the 215 females for which we only had one set of eggs, either pre, or post-mating supported the distinction of largely sexual versus largely asexual populations (Fig. 6). We found that the hatching success of virgin females is positively correlated to the sex ratio (i.e., the proportion of females) of their location (p-value < 0.001; Fig. 6A), while the hatching success of mated females does not depend on the sex-ratio of their location (p-value = 0.74; Fig. 6B). In addition, we found that mating increase significantly the hatching successes of females living with males, but not the hatching successes of females living without males in their location (p-value < 0.004, Fig. 6C).

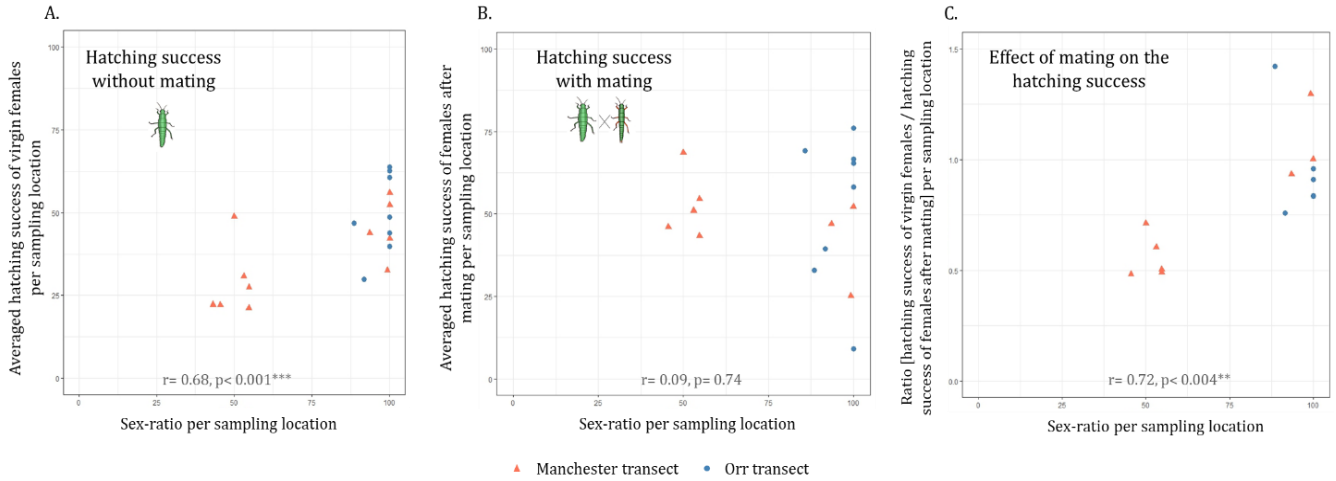


Figure 6. Hatching success of eggs depending of the local sex-ratio. A. Averaged hatching success of eggs laid by virgin females. B. Averaged hatching success of mated females. C. Ratio before/after mating hatching success, depending of the sex-ratio recorded in nature at each sampling location.

No overall difference in mating behavior of virgin females between locations

We then investigated whether some of the reproductive behavior of the females present in these different locations differed depending of the presence or absence of males living with them. We however found no correlation between the frequency or the duration of any of the recorded behaviors (i.e., guarding and mating) and the sex ratios of the locations (Fig. 7).

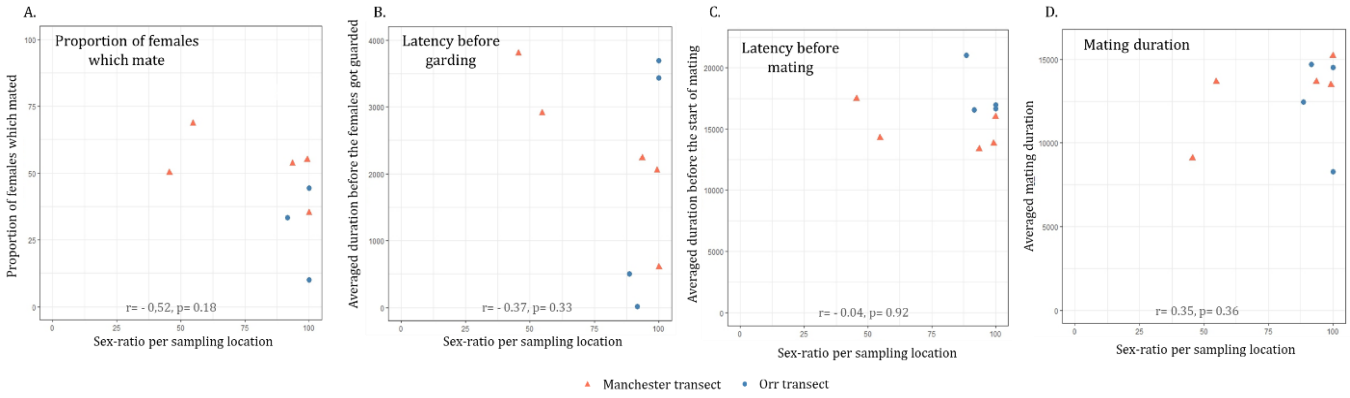


Figure 7. The mating behavior of virgin females do not depend of the local sex-ratio. (A). Percentage of females which mated, (B). Averaged time before a guarding behavior happened, (C) Averaged time before a mating happened, (D). Averaged duration of the mating, depending on the sex-ratio recorded in each sampling location.

DISCUSSION

In this study, we reported and characterized natural *Timema* populations with unusual sex ratios compared to the known and described obligate sexual and obligate parthenogenetic *Timema* populations. We show, for the first time in the *Timema* genus, populations containing a mixture of sexual and asexual individuals morphologically indistinguishable. In addition, this study suggests the existence of individuals capable of both modes of reproduction (i.e., facultative parthenogens).

Regarding the ability of virgin females to produce descendants parthenogenetically our results are clear and interpretable, but regarding the ability of these females to use sex to reproduce, on the contrary, additional analyzes are needed (see Perspectives section below) to demonstrate whether the eggs get fertilized or not. With our current data we can only assume that females with significantly higher reproductive success after mating are likely able to use sexual reproduction to reproduce.

First, the Manchester transect allowed us to highlight an area with a 50:50 sex-ratio followed very closely by an area with a 100:0 sex-ratio. Then, the Orr transect highlighted an alternation between locations containing 100% females and female-biased locations containing 9 to 23% males. Surprisingly, regarding the 50:50 and 100:0 sex-ratio locations, although these sex ratios are very close to expectations for obligate sexual populations (50:50) and obligate parthenogens (100:0), we found that none of them contain only sexual or only asexual individuals. On the contrary, both types of populations seem to be a mixture of sexual and asexual individuals co-occurring. They are constituted of very diverse females regarding their ability to reproduce asexually, ranging from a null

success to 100% hatching success in the 100:0 sex ratio locations, and from a null success to 70% hatching success in the 50:50 sex ratio locations (Fig. S1). We nevertheless found a positive correlation between the locations' sex ratios (i.e., proportion of females) and the parthenogenetic abilities. The more the locations were female-biased, the more they contain effective parthenogens. By contrast, mating with a male improved significantly the averaged hatching success of the females solely in locations containing males in the wild.

In the 100:0 sex ratio locations, we found a subset of females which had very low asexual abilities, but significantly higher hatching successes once mated (Fig. S1). They are thus likely able to use sex to reproduce. For this minority of females, we can hypothesize that, by inhabiting an environment where males are rare, they may usually fail to find a mate. In this case, they will not contribute to the sex-ratio of the population that will remain entirely constituted of females. A previous study using *Timema* regarding spontaneous parthenogenesis in sexuals (i.e., tychoparthenogenesis) have shown that an interaction between sex ratio and female mating probability could result in the loss of males, even when starting from a sexual population with an initially balanced sex ratio, and even if the rate of parthenogenesis was very low (Schwander *et al.* 2010).

In the 50:50 sex ratio locations, given the significant proportion of females with high asexual abilities, it is more difficult to understand how the population does not contain a female-biased sex ratio. Although it remains to be investigated, we can suggest two non-exclusive explanations that could lead to such a balanced sex ratio. One possible explanation would be that the sexual and asexual females differ in term of fitness under natural conditions, with the asexuals, despite a high reproductive potential, having a

reduced fitness compared to the sexuals (e.g., Lamb & Wiley 1979; Corley & Moore 1999). Another possibility is that these females, apparently facultatively parthenogenic, would reproduce exclusively using sexual reproduction if males are available to mate.

The last category of female biased locations that contained 9-23% of males is very unusual and unique compared to all the known and described *Timema* populations. These locations were found only along the Orr transect and were very close to locations containing no males. Overall, we found that they contained a high proportion of "*poor reproducers*" both pre and post-mating (40 to 50% of females from these locations were very inefficient in reproducing). By contrast, within the neighboring locations constituted only by females, we did not detect a single "*poor reproducer*". The presence of males in these locations thus seems to have a negative impact on the reproductive success of the females living among them. Our current data do not allow us to determine with certainty whether these males are functional and contribute in any way to reproduction within these locations. Determining the exact impact of these males within these areas thus remains a challenge for future research.

Interestingly, although this needs to be confirmed with certainty, our study also suggests that mating with a male can sometimes alter the reproductive success of asexual females. With our current data it is however not possible to certify that this result is not an effect of age. Indeed, since we allowed females to lay eggs before and after mating, the decreased reproductive success of females after mating is related to eggs laid by females a few days older compared to the eggs laid by virgin females.

Stick insects constituting the populations that we described in this study will be interesting for future research regarding evolutionary transitions toward parthenogenesis. In *Timema*, we know that facultative parthenogenesis (from virgin sexual females) and obligate parthenogenesis (from obligate parthenogens) proceed along different cytological mechanisms. The exact transmission of multi-locus heterozygous genotypes from females to their offspring strongly suggests that sexual species with some capacity for facultative asexual reproduction typically produce parthenogenetic eggs via automictic parthenogenesis (i.e., involving meiosis and fusion of two gametic products by central or terminal fusion; Schwander & Crespi 2009a; Fig. S3). By contrast, obligate asexuals reproduce via mitotic parthenogenesis (i.e., apomixis, Schwander & Crespi 2009a; Fig. S3) which is the most frequent mode of parthenogenesis in insects (Suomalainen *et al.* 1987). We can hypothesize that asexual lineages were initially reproducing by automictic parthenogenesis and then underwent a stepwise transition to apomictic parthenogenesis (White 1973; Bell 1982; Suomalainen *et al.* 1987; Castagnone-Sereno 2006; Schwander & Crespi 2009a). The change from sexual reproduction and fertilization to meiotic (automictic) parthenogenesis does not require any drastic departure in the cytological mechanism of meiosis (Fig. S3) as the fusion of nuclei from two different individuals is replaced by fusion of the nuclei within a single individual. The final step in such a transition to apomixis would then require the suppression of the first meiotic division (Bell 1982; Suomalainen *et al.* 1987). Moreover, if some rare sexual females are produce their eggs mitotically (White 1964, 1973; Bell 1982; Suomalainen *et al.* 1987), we can hypothesis that it would significatively favor transitions to asexuality. Very interestingly, in *Timema*, a previous study already found a single virgin sexual female (from *T. poppensis* species) which produced a large number of offspring via apomixis (Schwander & Crespi 2009a). The degree to which females of *T.*

poppensis, or of any of the other sexual *Timema* species, have the capacity for facultative apomictic parthenogenesis is therefore likely to be rare but still remains to be investigated at a larger scale. We can however hypothesize that if sexual *Timema* females have the potential for spontaneous apomictic parthenogenesis, then this process might favor transitions to asexuality and ultimately explain the large proportion of obligatory asexual lineages in this genus.

To conclude, our preliminary results suggest, for the first time in the *Timema* genus, the existence of facultative parthenogens. In addition to this we have highlighted locations where sexual and asexual individuals are cohabiting. Interestingly, our results indicate that these *Timema* populations include an extreme reproductive polymorphism. Ultimately, these results question the status of "species" for *T. poppensis* (previously considered as obligate sexual) and *T. douglasi* (previously considered as obligate asexual) which appears to be rather a mixture of individuals with very variable sexual and asexual abilities to reproduce. Such a reproductive plasticity may likely facilitates the success, spread and establishment of *Timema* in new localities and explain their very successful northward expansion and colonisation.

PERSPECTIVES

Ongoing analyzes; in collaboration with Guillaume Lavanchy

In order to clarify precisely the sexual reproductive abilities of the different *Timema* females which inhabit these regions, we are genotyping the females for which we know the hatching success of the eggs before and after mating (shown in Fig. 5), their offspring produced both from virgin and mated females, and the males that mated with these females. We are genotyping all these samples using next-generation RAD sequencing methods (protocol adapted from Brelsford *et al.* (2016) itself derived from Parchman *et al.* (2012)). Briefly, we are using restriction enzymes MseI and EcoR1 to digest genomic DNA, we are ligating barcoded adapters to digested DNA, amplifying each individual sample in four separate PCR reactions, pooling all PCR products and selecting fragments between 300 and 500 bp using agarose gel. Four libraries of 380 samples are currently sequencing on five Illumina (San Diego, CA, USA) HiSeq 2000 lanes at the Lausanne Genomics Technology Facility (Lausanne, Switzerland), producing single-end 125 bp reads. This analysis will enable us i) to know if the eggs produced after mating have been fertilized or not and thus to precisely quantify the sexual abilities of the females constituting these populations ii) to estimate the sex-ratio of the offspring, iii) to determine if the males found in the locations with unusual sex-ratios are functional and contributing to reproduction, iv) to study the transmission of multi-locus heterozygous genotypes from each mother to their offspring and thus to determine the cytological mechanism at the origin of the unfertilized eggs' development.

In addition to the genetic analysis of the individuals constituting these populations, we aim to estimate the reproductive success of the different females which live in these areas

under natural conditions. To this aim, we have installed 40 netbags in the field containing either a virgin female in areas inhabited exclusively by females, either a virgin couple in areas inhabited both by females and males. We will collect all eggs and estimate the fertility (i.e., both the eggs' production and the hatching success) under natural conditions of 40 females homogeneously distributed within the different sampling locations of this study.

Ultimately, the combination of these future works will allow us to conclude clearly on the asexual and sexual reproductive capacities of the different females present in these regions.

Acknowledgments

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SUPPORTING INFORMATION

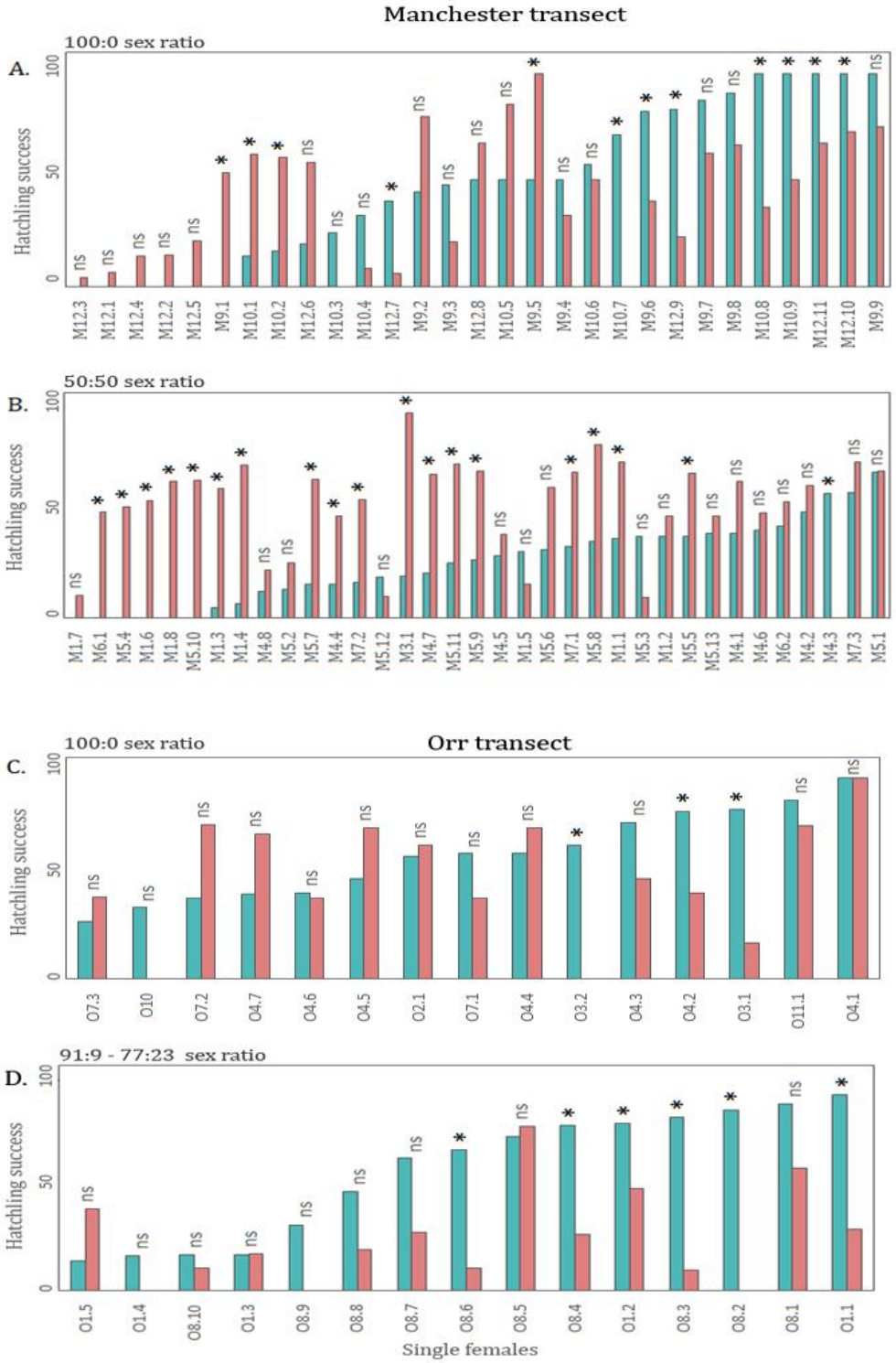

Figure S1. Hatching success of females before and after mating depending of the sampling location. For each female, the blue bar indicates its hatching success while still virgin and the red bar indicates its hatching success after mating with a male. Females are ranked according to the hatching success of their eggs laid when they were virgin (green bars). A and B show the two types of populations of the Manchester transect and C and D show the two types of populations found in the Orr transect. Stars indicate that for a given female, the hatching success before and after mating is significantly different ($p < 0.05$)

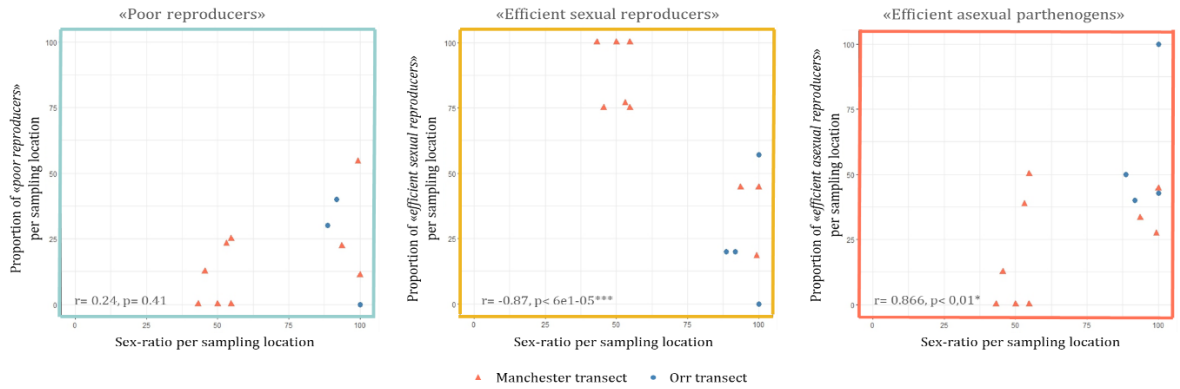

Figure S2. Locations with different sex ratios contain females with different sexual and asexual reproductive abilities. Symbols correspond to different sampling location. Shown is the proportion of females in each of the sampling location corresponding to A) poor reproducers, B) efficient sexual reproducers or C) efficient asexual parthenogens, as a function of the sex ratio in the sampling location. A Pearson's correlation test

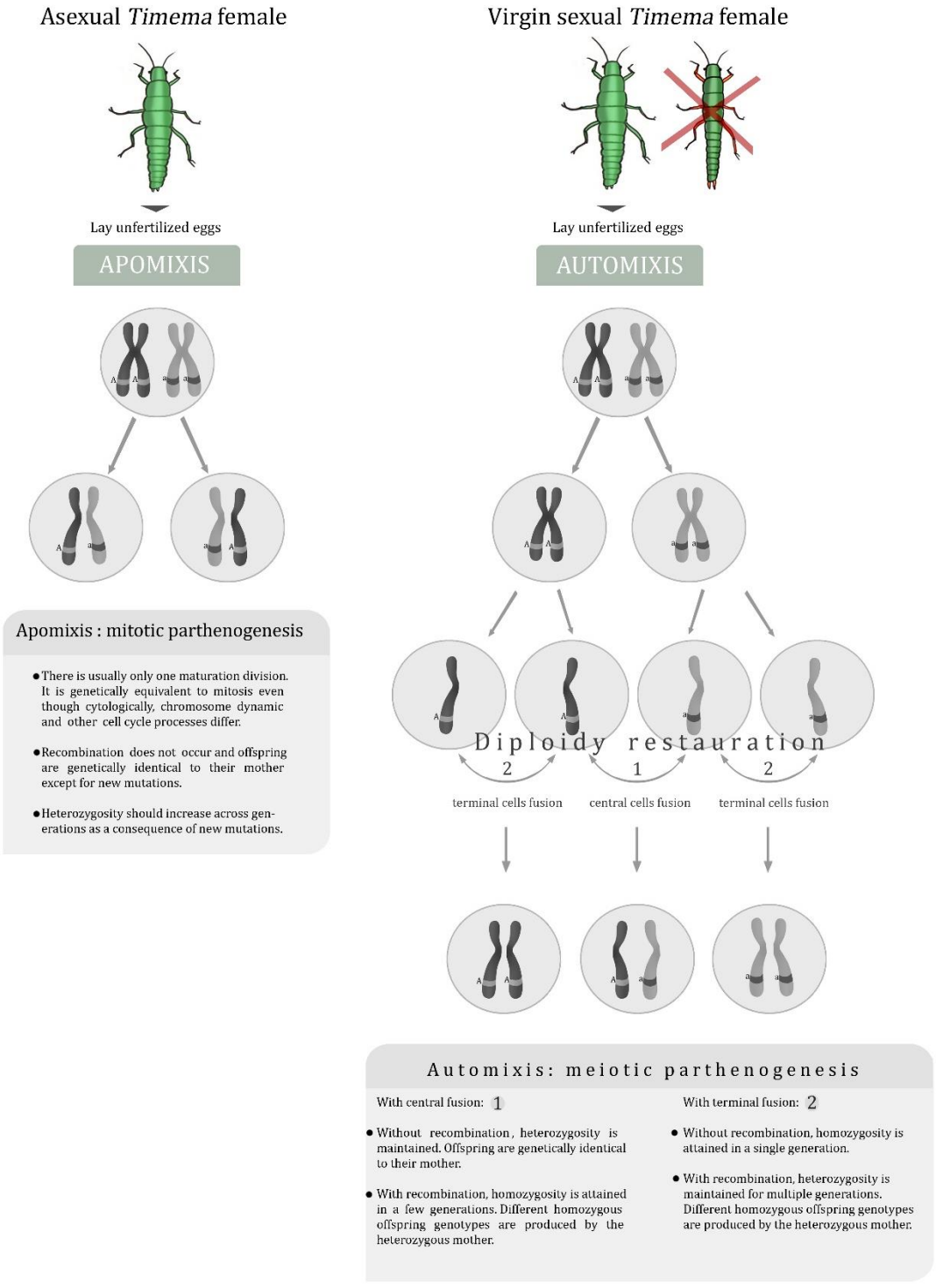

Figure S3. Mechanisms through which unfertilized offspring are produced by virgin sexual and obligate asexual females. Figure redrawn from Neiman and Schwander, 2011. The asexual females produce offspring via apomixis while the sexual females produce offspring via automictic parthenogenesis: with a relative proportion of oocytes produced either via central (1),or terminal (2) fusion.

- GENERAL DISCUSSION -

GENERAL DISCUSSION

In this thesis, my main objective was to contribute to answering the big and currently unresolved question "***Why do sex and males exist*?**" or more specifically "*Why has sex been the most common mode of reproduction in the living world for millions of years, while it seems so disadvantageous, costly and complicated compared to other reproductive modes, especially asexuality?*" (e.g., see Maynard Smith 1978; Kondrashov 2001; Agrawal 2006; Otto 2009). To investigate these questions, I chose to use sexual and asexual herbivorous stick insect lineages of the small genus *Timema*. This genus seems *a priori* ideal because it consists of several pairs of sexual-asexual sister lineages which allows us to make replicated comparisons. Only such a system with several pairs of sexual-asexual lineages will allow scientists to distinguish the effect of the reproductive mode from lineage specific effects. Throughout this thesis, I first attempt to clarify important aspects of the *Timema* stick insect ecology and evolution, and then empirically test hypotheses regarding the "paradox of sex".

In this brief general discussion, I first outline the main findings and contributions of my thesis to the knowledge of the ecology and evolution of *Timema*. I then follow by explaining why these aspects of the ecology of *Timema* are important and relevant to the empirical theories about the evolution of sex and asexuality that I ultimately aimed to test. Finally, I present the main conclusions about the consequences of asexuality in *Timema*, and I explain how these conclusions might contribute to a broader understanding of evolution and maintenance of sex in nature. Finally, I turn to several of the issues that have either not been answered by my research, or that are posed by the results presented here. I also mention the sorts of studies that could profitably be conducted on *Timema* to address general questions regarding the evolution of reproduction.

Main conclusions

The evolution of specialization in herbivorous stick insects

I aimed to investigate how niche breadths evolve following transitions from sexual reproduction to asexuality. To do so I first attempted to get a better understanding of the mechanisms and processes involved in the evolution of specialization. I first aimed to obtain an estimate of the degree of specialization of the different species of *Timema*, in term of host plant use (i.e., realized niche) and in terms of the ability to use hosts (i.e., fundamental niche). I then aimed to understand how specialization evolved and what processes underlie specialization in *Timema*, independently of the effect of the reproductive mode. I therefore initially focused solely on the sexual *Timema* species.

To date, most of the studies regarding specialization of herbivorous insects, including *Timema* stick insects, are based only on the number of hosts used in natural populations (i.e., the realized ecological niche; e.g., Colwell & Futuyma 1971; Nyffeler & Sterling 1994; Blüthgen *et al.* 2006; Slatyer *et al.* 2013; Forister *et al.* 2014; Rasmann *et al.* 2014). The fundamental niche of *Timema*, and thus the full range of diets allowing them to survive, grow and reproduce in the absence of predation and competition, was never studied until now. Regarding the degree of ecological specialization of *Timema*, I first reviewed the literature, which I then complemented with personal observations in order to characterize the realized feeding niche of the 23 known *Timema* species*. Timema* use plants from an unusually wide range of different families (including angiosperms and conifers) as hosts, with one to eight families of host plants per *Timema* species. In terms of realized feeding niche, the *Timema* genus thus comprises a range from relatively specialist through to generalist species (Chapter I). Furthermore, there is typically strong ecological specialization at the population level. That is, within a given population, stick insects favor the use of a single host species, even if other potential host species are available in large numbers around them (Chapters I and II).

A feeding experiment using nine sexual *Timema* species and seven *Timema* host plants further allowed me to characterize the fundamental niche breadth of these nine species. I then studied the evolutionary dynamics of the potential host plant ranges in a

phylogenetic framework. I found that stick insects which are ecological specialists at the species level (occurring on a single or very few plant species in natural populations) retain plasticity in host plant use and, surprisingly, feature broader fundamental feeding niches than ecological generalists. In line with classical theory, I found that specialization at the fundamental niche level comes at a cost of reduced ability to use non-native host plants. Conversely, species with a generalist fundamental niche showed little to no constraint in using multiple alternative host plants. Generalist fundamental feeding niches with specialist realized niches jointly evolved in the *Timema* species that shifted from angiosperm to conifer hosts. This is especially pronounced in those species that shifted to the redwood plant, which is chemically a very challenging host. The fact that the *Timema* living on redwood are ecologically very specialized despite their large potential niches indicates that ecological specialization is largely driven by factors other than feeding adaptations to host plants, for example competition and predation. I specifically found that the shift of *Timema* species to redwood host constituted a key feeding innovation that allowed these insects to expand northward in California while expanding their fundamental niche breadth (Chapter I). Overall, such fundamental feeding niche expansions following host shifts could facilitate future host shifts in the same lineage, which could in turn drive frequent host turnovers via positive feedback mechanisms. More broadly, these results improve our understanding of the evolutionary dynamics of host range expansion and contraction in herbivores.

Niche breadths of sexual and asexual organisms

Because asexual species derive from sexual ancestors, fundamental niches in new asexual species depend directly on the fundamental niche found in the ancestral sexual species. At the species level, a species may occupy a wide range of environments either because individuals are generalist in their habitat use, or because the species is made up of generally distinct individuals or populations, each specialized in its habitat use (Van Valen & Grant 1970). The *Frozen Niche Variation* model predicts that the phenotypic distribution of a new, recently derived asexual lineage would be narrower than that of its genetically variable sexual ancestor, because a single sexual genotype will be “frozen”, producing the new asexual clone (Vrijenhoek 1984; Case & Taper 1986; Case 1990; Weeks 1993). By contrast, the *General-Purpose Genotype* hypothesis (Lynch 1984 by see also

White 1973; Parker et al., 1977) proposes that asexual clones should generally have broader environmental tolerances and thus be more generalist than sexual individuals because of strong selection for phenotypic plasticity in asexuals.

I found that overall, sexual *Timema* species have a two to three times larger realized feeding niche than their asexual relatives at the species level and are therefore ecologically more generalist than asexuals in four out of five sexual-asexual species pairs (Chapter II). In the remaining pair, sexuals and asexuals use the same number of hosts. I also found that the degree of polymorphism of *Timema* at the species level is correlated to their degree of ecological specialization (Chapter II). Sexual stick insects may thus be able, thanks to a higher number of color morphs compared to the asexuals, to hide from predators on a broader range of host plants and substrates.

A feeding experiment focusing only on sexuals and asexuals from four *Timema* sister species pairs also allowed me to compare their fundamental niche breadths. Similar to the previous study regarding only the sexual species, I found that the size of the realized feeding niche of sexual and asexual *Timema* stick insects is not correlated to the size of their fundamental niche. Furthermore, the youngest asexual lineage tested (i.e., *T. monikensis*) is more specialist than its sexual relative, while the oldest *Timema* asexual lineage (i.e., *T. genevievae*) is more generalist than its sexual relative (Chapter II). This result may be of significance regarding the unusually old age of *T. genevievae*. Indeed, its "general purpose genotype could contribute to explaining its maintenance in the absence of sex over millions of years.

The study presented in this thesis is the first one that compares both realized and fundamental niche breadthes in replicated sexual-asexual lineages comparisons. It shows that such replicated comparisons are essential to draw general conclusions about the effect of the mode of reproduction. This study further suggests that young asexual species are more likely to have on average narrower niches, while older asexual species would feature broader niches than their sexual ancestors. Overall, the results improve our understanding of the ecological and evolutionary consequences of sexual and asexual reproductive strategies.

Interaction between Timema *hosts and their parasites*

I also aimed to test the *'parasite hypothesis for sex'* in *Timema*. I wanted to empirically determine if sex in this group could confer an advantage in countering parasitic pressures compared to asexuality. I first tried to know more about the diverse parasites affecting *Timema* stick insects, their ecology, and their evolutionary interaction with *Timema*, in order to ultimately choose a suitable system to test the *parasite hypothesis for sex*.

I have found and identified for the fist time three parasites of *Timema* stick insects (Fig 1). The two first parasites, some ectoparasitic mites and endoparasitic nematodes, were observed only very sporadically (< 1/500 infected *Timema* individuals). First, when sampling *Timema* in the field, some individuals were sometimes infected by generalist ectoparasitic mites (Fig. 1A). Given its rarity and non-specificity to *Timem*a hosts, I decided to not focus further on this ectoparasite in this thesis. Regarding the second parasite, I highlighted the existence of rare endoparasitic infections by mermithid nematodes that induced the death of their *Timema* hosts (Fig. 1B). This motivated me to study the evolutionary history and interactions of these nematodes with their *Timema* hosts. Finally, I found that a significant proportion of *Timema* stick insects from all sampled species and populations presented fungal infections that affected their fitness. I chose the fungi causing these infections for testing the *parasite hypothesis for sex*.

Figure 1. Pictures of *Timema* parasites. A. Ectoparasitic mites infecting a *T. cristinae* female in the field. B. endoparasitic mermithid nematode which exited and killed its *T. douglasi* host while emerging (See Chapter III). C. *T. californicum* female with melanized fungal infections (dark marks on its cuticle) after experimental infections performed in the lab (See Chapter IV)

Host-parasite coevolution is widely seen as a major driver of diversification and predicts co-diversification in hosts and their associated parasites. However, we found a complete lack of co-divergence between the endoparasitic nematodes and their *Timema* stick-insect hosts. By contrast, there was strong isolation-by-distance among the parasites, indicating that geography plays a more important role than host-related adaptations in driving parasite diversification in this system (Chapter III). My findings contribute to the growing evidence for lack of co-diversification between parasites and their hosts at macro-evolutionary scales (e.g., Cribb et al., 2001; Desdevises et al., 2002), which is in stark contrast with the overwhelming evidence for co-evolution within populations (e.g., Clarke, 1976; Price, 1980; Kiester *et al.*, 1984; Buckling & Rainey, 2002; Thompson *et al.*, 2005; Yoder & Nuismer, 2010; Ricklefs, 2010; Weber & Agrawal, 2012; Masri *et al.*, 2015). This highlights the need for studies linking micro- and macro-evolutionary dynamics in host-parasite interactions.

Test of the « parasite hypothesis for sex » in Timema

Using the fungi-*Timema* study system, I demonstrated that parasitic pressures are likely contributing to the maintenance of sex in the *Timema* genus. I first experimentally confirmed that fungal infections induced an immune response and had negative fitness effects for their *Timema* hosts. I also found that *Timema* hosts are locally adapted to the fungal parasites in the field, and that fungal parasites are transmissible between hosts. I found an overall "reproductive mode" effect since all asexual species were more infected than their sexual relatives in the wild. However, I found different pattern depending to the sexual-asexual species pair. In the *T. cristinae*/*T. monikensis* pair, I did not find evidence for the *parasite hypothesis for sex* and further investigation are needed. Indeed, in this species pair I found that both species seem tolerant to the fungal infections and that asexuals surprisingly are living in areas with higher parasite prevalence. By contrast, in the three remaining species pairs I found strong support for the parasite hypothesis for sex. In particular, I found that although parasite prevalence and parasite virulence are higher in locations of the sexual species, the proportion of infected individuals is smaller in sexual than in asexual populations. This indicates that sexual individuals are better able

to resist their local parasites than asexual individuals, while the asexuals are implanting and are maintained in areas where the parasite pressure is lower. Therefore, selective pressure from these fungal parasites seem to confer an advantage to sex over parthenogenesis in these three species pairs (chapter IV).

Interestingly, this study, similarly to the one in which I compared niche sizes of sexuals and asexuals, also shows that replicated comparisons are necessary if we aim to understand the ecological and evolutionary consequences of transitions to asexuality, without confounding lineage-specific effects. In addition, determining whether the capacity to exploit more ecological niches combined with the ability to better deal against fungal parasite pressures alone are sufficient to compensate for the costs of sex in *Timema*, or whether pluralist mechanisms are required (West *et al.* 1999; Neiman *et al.* 2017), remains a challenge for future studies.

Characterization of undetermined Timema *populations*

In parallel to the two questions that I tried to answer during my thesis (i.e., "*Is the ecological niche of sexuals and asexuals of different size?*" and "*Does sex confer an advantage against parasite pressures under natural conditions and through evolutionary time?*"), **I aimed to characterize in detail the reproductive strategies of two *Timema* species, *T. douglasi* and *T. poppensis*.** During *Timema* collecions in California, I discovered two geographical areas inhabited by *Timema* populations with unusual sex-ratios compare to the known and described sexual and asexual *Timema* populations.

The reproductive mode of *Timema* and consequencely the status of "species" has generally been inferred from the sex ratio recorded in the field. When the populations contained only females, they were described as "asexual populations" and when the sex ratios were close to 50:50 they were considered as sexuals. In the case of the populations I found, it was therefore impossible at first to know the mode of reproduction of the *Timema* individuals.

I sampled locations along two detailed transects, recording the precise sex-ratio at each location. For each sampling location along both transects, I then studied the hatching timing and hatching success of both virgin and mated females, as well as the mating behaviors of both females and males. My preliminary results indicate the existence of mixed populations where obligate sexuals and asexuals occur together, as well as the existence of facultative parthenogens, which was, to date, never found before in the *Timema* genus (chapter V). However, what the results mainly indicate is that these populations include an extreme reproductive polymorphism, and question the status of "species" for *T. poppensis* (previously considered as obligate sexual) and *T. douglasi* (previously considerred as obligate asexual) which appears to be rather a mixture of individuals with very variable sexual and asexual abilities to reproduce.

Some unanswered questions and perspectives

Ecology and evolution of sexual and asexual Timema stick insects

This thesis has improved our understanding of several aspects of the *Timema* stick insects' life history. However, there are still a number of gaps in our knowledge of *Timema* ecology, biology and evolution, that could, if they were filled, allow us to have a better understanding of the factors that maintain sex in this system.

- I aim to estimate *Timema* fitness in natural populations and specifically to determine precisely the reproductive successes of sexual and asexual species in the wild. It would be a major addition to our knowledge to have the fitness distributions of the sexuals and of their asexual relatives under natural conditions as it would allow us to enpirically quantify the direct costs of sex. Until now I did not succeed in obtaining the full picture, with the outputs still only partial (data not shown in the thesis report). This project is in progress.

- Because the distribution of sexual and asexual populations within a given sexual-asexual species pair does not generaly overlap in nature, future research needs to determine what exactly differentiates the environment inhabited by sexual populations from the environment inhabited by asexual populations. Despite differences in terms of

fungal parasite prevalence highlighted in this thesis (Chapter IV) to date there is no evidence of any major differences between zones containing asexual stick insects and areas containing sexual ones.

The cost and benefits of sex in Timema

▪ I also initiated a study (data not shown in this thesis) aiming to empirically test, in *Timema*, one of the major theoretical advantages of asexuality compared to sex, i.e., its putative "*twofold demographic advantage*" (Maynard Smith 1978; See introduction). I aim to infer their demographic history from molecular genetic data using coalescent-based methods, and to directly test whether asexuality generates a demographic advantage relative to sexuality. While preliminary results seemed to indicate a trend for all *Timema* populations to decline in the last tens of thousands of years, with a more pronounced decline in sexual populations than asexual populations, this project still requires extensive work to confirm this. Such a result, however, would confirm the demographic advantage conferred by an asexual reproductive mode. This project is in progress.

▪ We have obtained, with this thesis, a fine estimation of the degree of specialization both in term of fundamental niche and in terms of realized niche at the species and population level of asexuals compared to their sexual ancestors. Interestingly, we have highlighted the existence of recent highly specialized asexuals as well as generalist ancient asexuals regarding their ability to feed on a range of diverse host plants. This very generalist ability to use plants could be one of the features that allows the ancient asexual *T. genevievae* to be maintained over the course of evolutionary time, contrary to most asexual lineages which go extinct after a few dozen thousand years. However, our data do not allow us to relate the size of the ecological niche of asexuals to the clonal diversity which constitutes them. Theories such as the *Frozen Niche Variation* model which predicts that asexual clones would have on average narrower niches than sexuals, or the *General Purpose Genotype* which predicts that asexual lineages that would persist in the long term would be more generalist than their sexual ancestors (see Introduction) provide predictions regarding the scale of clonal lines. Indeed, a species may also be generalist because it would be made up of numerous clonal lines all specialized on a different fraction of the overall niche. One of the challenges of future research will be to clearly test these theories.

▪ Finally, regarding the *Parasite hypothesis for sex* in *Timema*, if we have already found evidence that *Timema* are adapted to their parasite community, and that the fungal parasite is able to transmit between its hosts, we do not yet know whether the parasite also adapts to *Timema*, and hence whether a co-evolutionary arms race is occurring. To address this, it is necessary to look for evidence of genetic adaptation by the parasite to the host, by measuring local parasite genetic diversity, assuming some form of genetic matching between parasite and host genotypes. Theory predicts that parasite diversity should be greater in local populations where the host is sexual, since sexual hosts should themselves show more genetic diversity. Conversely, if host genetic diversity would be lower in asexual host populations, parasites would need to be less diverse to infect them. Unfortunately, so far this project has been interrupted due to technical issues regarding molecular methods for extracting, amplifying and sequencing the DNA of the fungal parasite. One of the future objectives will be to develop a molecular method specifically to study this parasite or to find another way to collect and study the parasite directly from the field. For example, we could grow parasitic fungal strains directly from a living and just infected *Timema* host, before the fungi got encapsulate and melanized by the host, and then use Amplicon sequencing methods to sequence parasites and discriminate between the different fungal strains.

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APPENDIX

APPENDIX 1. Geographical distribution of the seven *Timema* host plants used in the feeding experiment (Chapters I and II) in the state of California, USA

The plant distributions are estimated with information from the public database USGS (https://bison.usgs.gov) and we used the QGIS software (QGIS Development Team, 2009) to get a visual representation of them. Stars indicate the 12 sampling locations of the *Timema* populations we used in this study.

APPENDIX 2. Other contributions during the PhD period (2013-2017)

Scientific research

- Fieldwork collection of *Timema* stick insects in California, U.S, two to three months at the spring of each of the years 2013, 2014, 2015, and 2017.
Contribution to the research projects of all students and researchers working on *Timema* in the Schwander team during these years (future co-authorships).

- Early supervision (labwork, sequence analyzes, software explanations, weekly discussions) of Amaranta Fontcuberta during her Master thesis, 2015.
Conribution to the research article: Fontcuberta García-Cuenca, A., Dumas, Z., & Schwander, T. (2016). Extreme genetic diversity in asexual grass thrips populations. *Journal of evolutionary biology*, *29*(5), 887-899.

- Supervision of Simone Ariëns Master thesis, 2015.
"Demographic consequences of sexual and asexual reproduction in *Timema* stick insects": Project still in progress.

Scientific outreach

- Co-conception and co-realization with Tanja Schwander of a workshop and several posters for the exhibition "LAB-LIFE - Exploration du vivant", at "Le musée de la main", of Lausanne, autumn 2014.
Title: Bête comme une mouche ? Testez la capacité d'apprentissage des mouches du vinaigre.

- Member of the organizing committee of the Biology'16 conference event (Lausanne, February 2016). Involved in various tasks related to the organization, the conception of the scientific program, the selection of abstracts for talks, flash talks and posters.

- Co-organizer (with Dr. Tania Jenkins and Dr. Lucie Froissard) of the "Speed Dating Scientifique", a science outreach event held in parallel of the Biology'16 conference.

APPENDIX 3. Poster conference Jacques Monod, Roscoff (2013)

"Recent advances on the evolution of sex and genetic systems"

Understanding the transition from sexual reproduction to parthenogenesis

An experimental evolution of new parthenogenetic lines

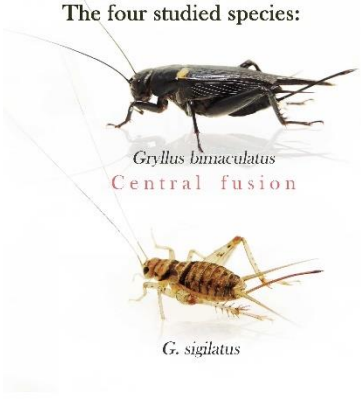
Larose Chloé & Schwander Tanja
contacts: chloe.larose@unil.ch ; tanja.schwander@unil.ch

Understanding the evolution of novel traits is often hampered by a lack of information on intermediate stages in transitions from ancestral to derived phenotypes. The aim of the present study is to develop insights into transitions from sexual reproduction to parthenogenesis. Using an experimental selection approach in criquet and grasshopper species, I want to evaluate whether obligate or facultative parthenogenesis may evolve from spontaneous parthenogenesis.

Spontaneous parthenogenesis: Spontaneous development and hatching of a small proportion of unfertilized eggs in a normally sexually reproducing species

Question : Could spontaneous parthenogenesis, known to occur in many sexually reproducing species, provide a stepping stone from sexual reproduction to parthenogenesis ? Are certain cytological mechanisms underlying spontaneous parthenogenesis more efficent than others?

The four studied species:



Gryllus bimaculatus
Central fusion

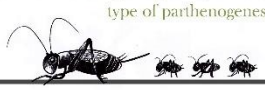
G. assimilis

G. sigilatus

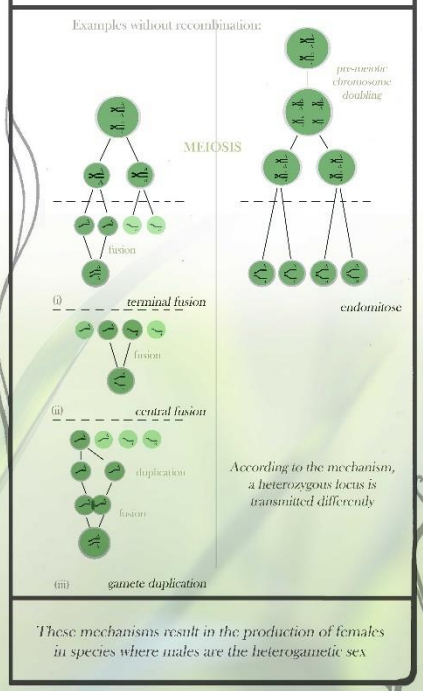
Locusta migratoria
Gamete duplication

Mitotic or meiotic parthenogenesis?

Spontaneous parthenogenesis is presumed to occur because 'errors' during the meiotic divisions can result in the production of diploid instead of haploid oocytes (White, 1973). It is known to always happen by a meiotic type of parthenogenesis.



Cytological mecanisms of diploidy restoration in meiotic parthenogenesis



These mechanisms result in the production of females in species where males are the heterogametic sex

An experimental selection to increase spontaneous parthenogenesis

Protocol:

- Create a genetically diverse base population by mixing several geographically distant populations, in each species
- Evaluate the spontaneous parthenogenetic potential of each species
- Conduct experimental selection to increase and decrease rates of spontaneous hatching
- Identify and explore the correlated responses that these changes might entail
  -> generation time, longevity, life-time egg production, developmental constraints, ...

Objective and discussion:

This project could help to better understand the transitions between reproductive systems, and therefore their diversity, and their evolution. It may provide new insights into the maintenance of sex in natural populations and help to explain why facultative parthenogenesis is rare in animals.

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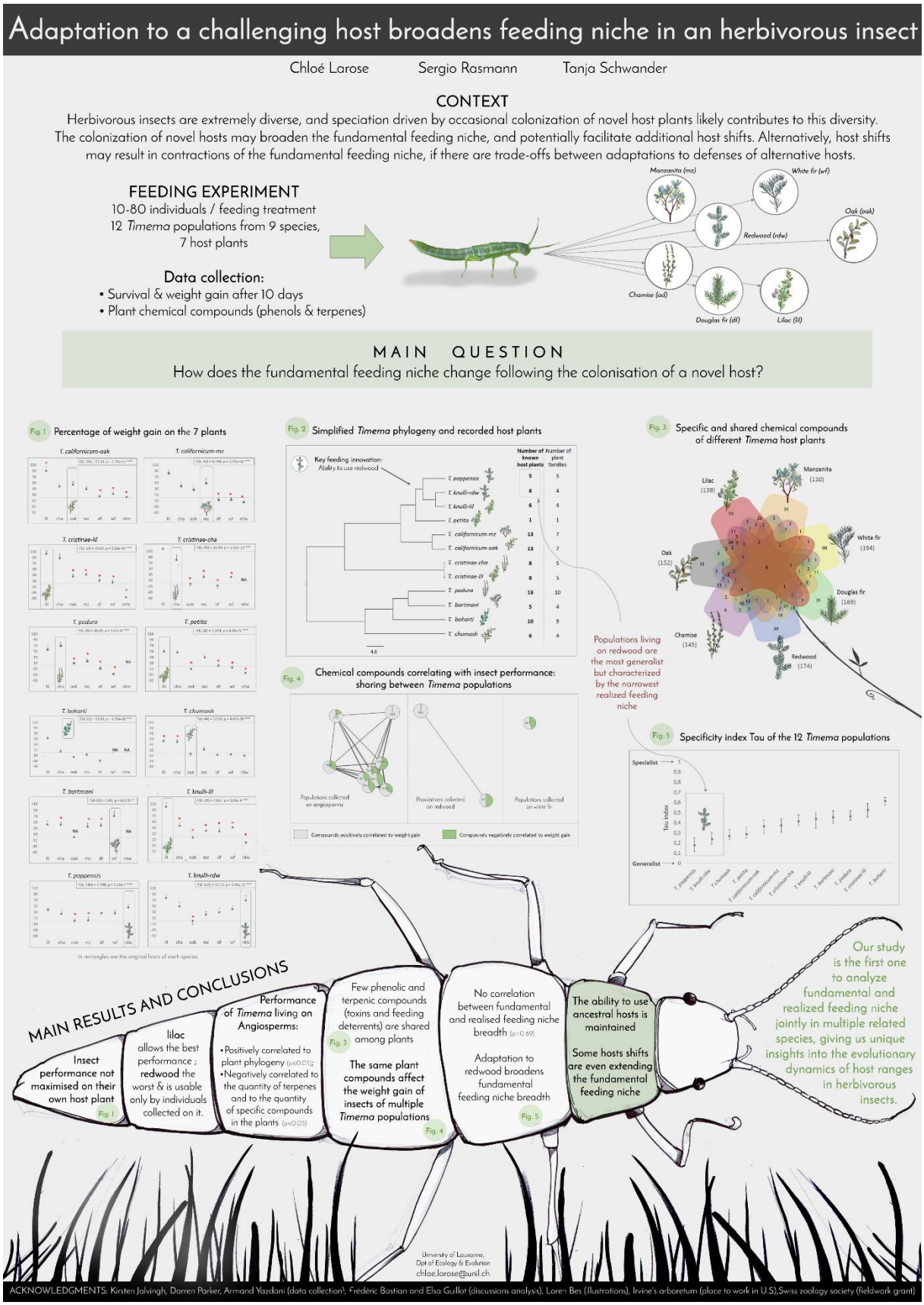
Department of ecology and evolution

APPENDIX 4. Poster conference ESEB (2017)

Symposium "Evolutionary dynamics"



APPENDIX 5. Curriculum vitae

La rose Chloé

Born 09. 01. 1988
Chloé Larose
Université de Lausanne,
DEE - bâtiment Biophore
CH-1015 Lausanne
Suisse (Switzerland)
Tel: +0041.76.682.77.36
chloe.larose@unil.ch

Member of the Genetics society since 2014.
Member of the Swiss zoological society since 2015.
Member of the Society for the study of evolution since 2015.

2013 Started the PhD project: Study of the processes underlying the transition from sexual reproduction to asexuality.
with T. Schwander (Department of Ecology & Evolution, University of Lausanne , Switzerland
2012 (april to august) Volunteering in the Kanahau association (Utila bay island, Honduras)
2012 (january to march) Volunteering in the Utila Iguana research & breeding station (Utila bay island, Honduras)
2011 (january to august) Study of genomic regions subject to divergent selection between sexual and asexual lineages of the aphid Acyrthosiphon pisum with S. Stoeckel & C. Rispe (INRA, Le Rheu (35), France)
2010 (january to april) Chemical ecology of the ladybird Coleomegila maculata.
with J-L. Hemptine & A. Magro (National School of Agronomy, Ramonvilles (31), France)

Conferences

2017 " ESEB" (University of Groningen, Netherland)
Poster: "Adaptation to a challenging host broadens fundamental feeding niche in an herbivorous insect"
2016 " Conference Biology 16" (University of Lausanne, Switzerland)
- Member of the organizing committee - Involved in various tasks related to the organization, the selection of abstracts for talks, flash talks and poster.
- Co-organizer of the "Speed Dating Scientifique", a science outreach event held in parallel of the Biology 16 conference.

2015 " ESEB" (University of Lausanne, Switzerland) - Talk: "No evidence for parasites maintaining sex in natural stick insect populations"
2014 " Conference Biology15" (EAWAG institute, Switzerland)
Talk: "No evidence for parasites maintaining sex in natural stick insect populations"
2013 " Conférence Jacques Monod - Recent advances on the evolution of sex and genetic systems" (CNRS, Station Biologique de Roscoff, France)
Poster: "Transition from sexual reproduction to parthenogenesis - An experimental evolution of new parthenogenetic lines"
2011 " Conférence Le petit pois déridé " (Natural History Museum of Toulouse, France)
Talk: "Effect of reproductive mode on the structuring of genetic diversity across genomes and genes sequences targeted"

Publications

- Larose C, Rasmann S and Schwander T. (2018) Evolutionary dynamics of specialization in herbivorous stick insects, submitted to *Ecological Letters*
- Larose C and Schwander T. (2016) Nematode endoparasites do not codiversify with their stick insect hosts. *Ecology and Evolution* 6(15) pp. 5446-5458.
- Jaquiéry J, Stoeckel S, Larose C, Nouhaud P, Rispe C, *et al.* (2014) Genetic control of contagious asexuality in the pea aphid. *PLoS Genet* 10(12):e1004838.

Education

2009-2011 Master 2 EFCE: Fonctional, Behaviorial, and Evolutionary Ecology (University of Beaulieu, Rennes (35), France)
Master 1 Ecology (University of Paul Sabatier, Toulouse (31), France)
2006-2009 Lience BOPE: Biology of Organisms, Populations, and Ecosystems – Facultative option: Course of "Universe/space exploration" (University of Paul Sabatier, Toulouse (31), France)
2005-2006 Scientific Baccalaureat – Speciality Maths – Option Arts (Bellevue High School, Albi (81), France)

Interests

Member of the association "Nature et Midi Pyrénées", Nature protection, Toulouse, France (2009, 2010, 2011).
Member of the association "AFEV", Assisting young students with difficulties, Toulouse, France (2006, 2007).
Member of the association "Les resto du coeur", Toulouse, France (2006, 2007, 2008).

Competitive grants

2015 Students travel grants from the Swiss Zoological Society
2014 Heredity Fieldwork Grant from The Genetics Society

CV