Inferring Evolutionary Population History: Katydid And Fish Case Studies

Nathan W. Bailey

Thesis submitted for the degree of doctor of philosophy, University of St. Andrews

January 2006

DECLARATIONS

I, Nathan W. Bailey, hereby certify that this thesis, which is approximately 29,000 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

Date 13 March 2006 Signature of candidate

I was admitted as a research student in September 2002 and as a candidate for the degree of Ph.D. in September 2003; the higher study for which this is a record was carried out in the University of St. Andrews between 2002 and 2005.

Date 13 March 2006 Signature of candidate

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate for the degree of Ph.D. in the University of St. Andrews and that the candidate is qualified to submit this thesis in application for that degree.

Date 13 March 2006 Signature of supervisor

COPYRIGHT

Date 13 March 2006 Signature of candidate

ABSTRACT

I used mtDNA sequencing and microsatellite analyses to elucidate and provide statistical support for models of population history in katydid and fish study systems. Mormon crickets are distributed throughout western North America and display two phases (solitary and gregarious) that have been assumed to be phenotypically plastic. Solitary populations occur on the eastern slope of the Rocky Mountains, whereas migratory gregarious bands typically occur west of the continental divide. Solitary animals are cryptically coloured and sedentary, but gregarious ones are aposematically coloured, form migratory bands and show sex role reversal. 1) We tested the hypothesis that there is no genetic division between solitary and gregarious populations. Mitochondrial gene sequencing using COII and COIII revealed two clades in Mormon crickets that unexpectedly correspond with the solitary-gregarious distinction but also with geography. 2) Sequence analysis and coalescent modeling show that these clades diverged approximately 2 million years ago and represent discrete lineages. 3) Partial Mantel tests indicate that phase is loosely correlated with the genetic division but does not best predict it, and our results taken together give much better support to the hypothesis that the Rocky Mountains acted as a barrier during Pleistocene glacial cycles, driving populations into separate east-west refugia. 4) East-west phylogroups uncovered in a median-joining haplotype network show remarkably different patterns of population genetic structure, especially in isolation by distance, which is most likely due to the different dispersal abilities and life history differences between solitary and gregarious phases, which are largely coincident with the genetic clades. The experiences of the two refugial clades during the Pleistocene glaciation/recolonisation cycles was dissimilar, with the western phylogroup bearing the genetic signature of a marked historical population expansion, as inferred from analysis of mismatch distribution. 5) Solitary and gregarious male Mormon crickets show differences in their reluctance to sing and in calling song and the morphology of sound producing organs. In particular, the relationship between mirror size and carrier frequency is different between the two phases. Thus in Mormon crickets, patterns of recent population differentiation dating to the Holocene and influenced by life history and dispersal differences are superimposed on an earlier genetic subdivision arising in the Pleistocene, indicating markedly different evolutionary histories for two refugial clades that are largely coincident with phase. These differences are consistent with subspecies.

The Goodeid fish species *Zoogoneticus tequila* and *Ameca splendens* are endangered and threatened (respectively) in the wild where they exist in small populations. "Ark" populations of both have also been kept in aquaria for several decades. 6) Aquaria populations are expected to show reduced genetic diversity, but has genetic diversity been reduced in the wild as well? Severe reductions in microsatellite diversity indicate significant inbreeding in ark populations of both species, and such reductions can occur over very short timescales in ark populations even if they are well managed. A Bayesian approach indicates that the wild *Z. tequila* population has experienced a recent, severe population decline, and likely has a high risk of extinction as a result of increased potential for inbreeding.

ACKNOWLEDGEMENTS

I really appreciate all of Tanya Hamill's help, and Leon Hockham passed on this wise bit of information (a similar quote may have appeared in his own thesis): "never mind the contamination in the negative control." These are words to live by.

With the patience of Job, my friends tolerate my mercurial mood swings and bizarre tendencies. Ruth, Dave, Mary and I avoided getting gout on our Pyrénéen field trip despite our overindulgence in cheese and wine. Jenny, Jeff, Kit and Susi always seemed to be by my side when life's little dramas erupted. Aoife is my best-ever, favourite flatmate. Recalling her lying pissed, half in the flat, half out of the flat, hilariously screaming compliments to herself and barking commands at me has helped me through many a dark moment. Gordon, Paty, Kirsten, Joe and Valentina are stabilizing forces; the long-suffering inertia that keeps me from aimlessly flapping out of control both in my scientific endeavours and in my bipolar social life. I will miss our endless talks about all matters biological, and our fanatical adherence to the Thursday night pub quiz ritual. Łukasz's intelligence and aptitude for evolutionary biology is scary, though it would take a US-style extraordinary rendition to make him admit it. He has evangelized Dawkins to me and given honest advice on various aspects of my research. Dla Ciebie, mógłbym zrobić wszystko; co zechcesz powiedz tylko; naprawdę na dużo mnie stać!

I am glad to have been able to bounce ideas off the knowledgeable Joe Tomkins and Jeff Graves. Tino Macías Garcia has infused his enthusiasm into the Goodeid fish project and is a friend with whom I have had many interesting conversations about sexual selection and evolutionary biology. Darryl Gwynne has been a great resource throughout this PhD, from extending his hospitality during my visit to Toronto to generously sharing his considerable knowledge during fieldwork. I have really enjoyed working with him.

Three and a half years ago, I had never set foot in Scotland, had no friends here, didn't know what I was going to work on, and had certainly never met this Mike Ritchie guy. I'm glad I did, though, and if anyone is to be thanked it is Mike: for tolerating my insane work schedule, supporting my cockamamie scientific ideas, fostering my scientific development (maybe not as fast and extensive as it should have been!), being privy to all my slimy dark secrets, and above all for giving me the leeway and unconditional support to develop this PhD in a variety of truly fascinating directions. I look forward to a future of collaborations, if I should be so lucky.

I have received nothing but wise, good-natured support from my entire extended family, especially Ann, Anna, Jean, and my grandparents. Sarah, my mom and my dad are the three flying buttresses propping up this wobbling jelly of a life. I cannot think of a better, fitter person with whom to share fifty percent (statistically speaking) of my genes than Sarah. I also have the only mom who bakes cookies, brownies, cakes and candies and then ships them three thousand miles across the Atlantic to ensure that I retain a thick layer of blubber (talk about parental care—Trivers would be impressed). Finally, my father was the first, and will always be the most influential, scientist in my life. My love and curiosity of science and the natural must have started with our forays into the forest searching for fungi...I recall the stunning (reducible!) complexity of those weird inhabitants of the litter: Destroying Angels hatching out of their disingenuous little eggs, dusty Puffballs, Shaggy Manes, saprophytic Lobster Tails, Fly Agarics and Amanitas, Corpse Finders, those festering phalluses of putrefaction the Stink Horns, and of course my favourite—the Stinky Squid. Now here is a story about an equally bizarrely named creature, the Mormon cricket, with an equally bizarre life history…

CONTENTS

GENERAL INTRODUCTION

Preface: elucidating evolutionary histories

The research I describe here is wide-ranging and explores how the evolutionary history of species or populations can be elucidated using genetic markers and behavioural studies. I have primarily made use of an insect study system, but I have also included a chapter on historical demography of wild and captive "ark" populations in two species of endangered Goodeid fish. The appendix was written as a review paper to investigate the question of whether sexual selection theory can predict homosexual male partner choice. Each chapter was written as a manuscript with its own introduction; therefore, the general thesis introduction and conclusion focus almost solely on Mormon crickets and attempt to provide information that is not already included in the data chapters.

My research addresses two broad questions:

1) Can we test *a priori* hypotheses about historical evolutionary processes using genetic data from contemporary populations and statistical phylogeography?

2) Which factors are most likely to have influenced patterns of genetic differentiation in the study species: isolation in refugia, dispersal ability, life history differences, or mating system?

Mormon crickets: a historical perspective

During the mid to late 1840s, Mormon settlers (most of whom were European converts) on the western frontier of the United States were locked in a cycle of settling new territories only to be driven further west by citizens angered by their unorthodox religious teachings and practices (Krakauer 2003). A mass migration eventually brought them westward through northeastern Colorado. They cut across the formidable Rocky Mountains via southwestern Wyoming (circumventing a commonly used trail through the Poudre Canyon in Colorado), and eventually halted in the Salt Lake Valley in Utah in 1847 (MacVean 1987). There they

encountered another migratory species to which they gave their own name: the Mormon cricket (figures 1-8).

An outbreak of Mormon crickets (*Anabrus simplex* Haldeman 1852, Tettigoniidae, Tettigoniinae, Platycleidini) in the following summer inflicted significant damage to the crops of Mormon settlers in the Salt Lake Valley, but according to legend the crickets were decimated by seagulls, thus elevating seagulls to "state bird" status (MacVean 1987, utah.com 2005). Accounts of early outbreaks may be subject to some slight creative license, with vivid descriptions such as: "[Mormon crickets] swept down from the surrounding mountains and threatened complete destruction of crops and the ultimate starvation of many of the settlers" (Cowan *et al.* 1943). Nevertheless, severe Mormon cricket outbreaks have been documented with regularity since the settlement of Utah, with sporadic reports of damaging outbreaks across the Western US throughout the latter half of the 1800s and the early 1900s (Cowan and Cambell 1929, Cowan 1933, Schweis *et al.* 1939, Cowan *et al.* 1943). A particularly devastating episode of Mormon cricket activity started during the Great Depression and peaked in the late 1930s, causing significant crop and rangeland damage throughout western states (Cowan 1933, Mills and Hitchcock 1938, MacVean 1987, Anonymous 1991). Recent Mormon cricket outbreaks have been documented in the 1980s (MacVean 1990, New York Times 1990) and in the last several years Mormon cricket densities have reached those last seen during the 1930s (Associated Press 2003, Brown 2004).

It is therefore unsurprising that the vast majority of research and publications on Mormon crickets is focused on either their control or their ultimate elimination from the rangeland ecosystem (eg. Cowan and Campbell 1929, Cowan 1933, Mills and Hitchcock 1938, Schweis *et al.* 1939, Sorenson and Jeppson 1940, Cowan *et al.* 1943, MacVean 1987, Redak *et al.* 1992, Lange *et al.* 1995, O'Neill and Turnbow 1999, Pace 2003, Brown 2004). Although some studies have focused on using naturally-occurring parasites of Mormon crickets as a means of reducing impact on crops and rangeland (Lange *et al.* 1995, O'Neill and Turnbow 1999), most control methods rely on the use of pesticide applications (Pace 2003, Brown 2004). Work in the latter half of the last century has indicated, however, that Mormon crickets may not be as damaging to rangeland as previously assumed; they have been held responsible for the major economic impacts and habitat destruction of the Great Depression outbreaks when in fact the effects of these outbreaks may have been greatly exacerbated by overgrazing and extreme drought

(MacVean 1987 and references therein, Redak *et al.* 1992). Native Americans in the region had co-existed with Mormon crickets and developed more innovative uses for them and other outbreak species (Madsen 1989). Mormon crickets have a high fat content (16%), and Native Americans collected and ate them dried or in cakes (Madsen 1989). Mormon crickets have also been suggested to be a high-quality source of nutrition for inclusion in high-protein livestock feed (DeFoliart *et al.* 1982).

Synopsis of recent Mormon cricket research

Attention has only recently shifted from the allegedly destructive powers of Mormon crickets to some of the more intriguing aspects of their life history. Gwynne (1981) first investigated Mormon cricket mating behaviour in the context of sexual selection theory (although very early investigators did note the peculiar "hominy" donated by males to females; eg. Gillette 1904, Snodgrass 1905), and subsequent investigations have focused on two other areas: investigating how outbreaks may be effected by biotic and abiotic cues (eg. Sword 2005) and characterizing movement patterns in migratory bands (eg. Lorch and Gwynne 2000, Lorch *et al.* 2005).

Mormon crickets do not only occur in destructive migratory bands, however. Populations may also solely consist of cryptically coloured sedentary individuals that differ in both morphology and behaviour (colour in these populations is much more variable, ranging from emerald green to dark brown; see figures 3 and 5). Field observations suggest that once all individuals in such a population have matured to adulthood, a migratory outbreak is unlikely to occur (pers. obs.), although no rigorous study has confirmed this. Surprisingly, no genetic work had been done on Mormon crickets, and the expression of the "outbreak" versus the "non-outbreak" phenotype was assumed to be facultative, i.e. a phase polyphenism that is induced by environmental differences as opposed to genetic differences. This has been likened to the more well-known phase polyphenisms in migratory locusts such as *Schistocerca migratoria* and *S. gregaria* (MacVean 1987, Gwynne 1984). In the latter species, changes in population density have been shown to mediate the expression of the aposematically coloured outbreak, or "**gregarious**" phenotype in juveniles, as opposed to the cryptically coloured non-outbreak, or "**solitary**" phenotype occuring under low density regimes (Simpson *et al.* 2001, Sword and

Simpson 2000). I will use the "gregarious" and "solitary" designations to refer to migratory, band-forming Mormon crickets versus sedentary Mormon crickets throughout this thesis for consistency; however, Sword (2005) discusses the semantic justifications for using either term.

The solitary and gregarious phenotypes of Mormon crickets have been observed to correlate with geography: solitary populations studied by Gwynne in the 1980s were primarily located on the eastern slope of the Rockies, whereas the gregarious populations he studied were west of the continental divide in northwestern Colorado and Utah. Despite the lack of genetic evidence, the morphological and behavioural differences between these populations have been assumed to be plastic responses to varying biotic and abiotic environmental conditions in their resepective geographic locations. For example, Gwynne (1981, 1984) demonstrated that sex role reversal occurred in high density (or gregarious) western populations, with females competing aggressively for matings with discriminating males, and suggested that "...the expression of sexual differences in behaviour is, to a large degree, facultative" (Gwynne 1984). Further experiments showed that sex role reversal can be mediated by diet. Gwynne (1993) assessed courtship behaviour in replicate cages of Mormon crickets supplemented with either high-quality food (high protein food items) or low-quality food (low protein food items). Sex role reversal and increased sexual selection for female size was observed in the low-quality food, but not the high-quality food replicates, suggesting that courtship role reversal was a plastic trait mediated by diet quality (Gwynne 1993).

While this research demonstrated the potentially facultative mechanism underlying sex role reversal in Mormon crickets, it did not address the broader morphological and behavioural differences between the solitary and gregarious animals. MacVean (1987) stated that,

> "It is not understood how or why a transition occurs from widely scattered, sedentary individuals to densely-aggregated, migratory bands of insects...The process bears a striking resemblance to phase transformations in the African plague locusts, *Schistocerca gregaria* (Forskal) and *Locusta migratoria migratorioides* (Reiche and Fairmaine)."

To a large degree, the cues required for the induction of gregarious behaviour have remained uninvestigated until only very recently, and the term "phase" has been applied to the solitary and gregarious forms of Mormon crickets without solid evidence that these forms are in fact true

expressions of a phase polyphenism. I will continue to refer to them as phases thoroughout the thesis for consistency; however the appropriateness (or not) of this designation should be borne in mind.

The only research published to date that addresses MacVean's question investigated the effects of long-term rearing densities and short-term presence of conspecifics in effecting gregarious-like behaviour, and found that high long-term rearing densities only influenced the turning behaviour of Mormon crickets (high rearing densities induced greater turn angle, for example) whereas short-term inter-individual interactions were more likely to cause movement patterns typical of gregarious bands (Sword 2005). Inter-individual interactions were achieved by exposing a focal insect to four conspecifics, and this treatment correlated with greater walk time, speed, and distance in the focal insect (Sword 2005). The exact nature of the inter-individual interactions responsible for this change in behaviour was not tested; however, this conclusion mirrors results in *S. gregaria* in which tactile stimulus on the hind femurs of the insects induced gregarious behaviour (Simpson *et al.* 2001). While this appears to lend support to the assumption that—as in *S. gregaria*—solitary and gregarious Mormon crickets display a phase polyphenism in which inter-individual interactions induce gregarious migratory behaviour, it is impossible based on the present evidence to exclude other explanations for these remarkably different phenotypes, for example, that they may simply represent genetic differences accumulated during separate evolutionary histories.

While the different natural histories of solitary and gregarious Mormon crickets are well-described in the literature (eg. MacVean 1987), the ultimate causes of those differences is still relatively untested. For example, Gwynne's (pers. comm.) and my own field observations suggest that upon reaching adulthood, all individuals in a gregarious band retain the aposematic gregarious phenotype throughout their lives, although stragglers left in low densities in the wake of a band will adopt normal courtship roles whereupon males perch and call to attract choosy females—a situation more similar to the normal courtship roles seen in solitary populations. This indicates a degree of behavioural plasticity (that may be related to density, or more proximately, the likelihood of encountering conspecifics) in gregarious individuals, even though such solitary-behaving stragglers cannot change their aposematic colouration. Solitary individuals in populations on the eastern slope of the Rockies, however, have never been observed to switch (at any time during their development) from the solitary to the gregarious

phenotype. This does not exclude the hypothesis that they, too, can change from one phase to the other; but suggests that if the phenotypic differences are solely determined by the environment, the environmental conditions favouring the solitary phenotype have remained relatively consistent in populations on the eastern slope of the Rockies. Unfortunately there has been no formal investigation of habitat differences between solitary and gregarious sites, although field observations suggest that solitary populations are more likely to be found in rich meadow habitats with numerous floral and grass species (see Gwynne 1984), whereas gregarious populations have been seen moving through numerous habitat types including sage brush, juniper-forested hills and recently (ca. 1-5 years) burned forest (Sorenson and Jeppson 1940, pers. obs.). It is unknown whether these habitat types directly influence the expression of the gregarious versus solitary phenotype, or whether the differences in habitat provide differing natural selection pressures for the Mormon crickets inhabiting them, or both. Radiotracking transplantation experiments indicated that the aposematic gregarious phenotype confers a selfish-herd advantage in gregarious bands moving through dry sage brush habitats—gregarious Mormon crickets tracked within bands suffered far lower mortality than conspecifics transplanted outwith the band (Sword *et al.* 2005; see figure 7)—however no experiment has been carried out to determine the relative advantage of the cryptic colouration of solitary individuals.

Given that the solitary and gregarious phenotypes in Mormon crickets have been assumed to be phase polyphenisms and so little is known about the environmental cues that mediate switches between the putative phases, it was logical to proceed with genetic studies. The first was designed to test the easily falsifiable hypothesis that the solitary and gregarious distinction in Mormon crickets may be a result of genetic differences between the two forms . Rejection of this would lend support to the idea that the solitary and gregarious phenotypes of Mormon crickets represent a true phase polyphenism, and further work could elucidate how and when the different phases arise. As with most scientific endeavours, however, it was not so easy. This thesis describes how the testing of this first simple hypothesis led to a much more detailed and complex investigation of the evolutionary history of Mormon crickets.

In line with the general aims of this thesis, the specific goals of the Mormon cricket component of my research were to:

(1) Develop a statistically supported model of the evolutionary history of Mormon crickets, with particular emphasis on the role of phase, geography and life history in predicting the geographic distribution of genetic variation within this species.

(2) Extend Gwynne's (1981, 1984, 1993) sexual selection work by investigating male song and reproductive isolation between normal and sex role reversed populations of Mormon crickets.

Phylogeography: the evolution of a discipline

> "There are two essential steps in phylogeographic analysis: (i) the generation of a gene genealogy; and (ii) the interpretation of this genealogy." –Lowe *et al.* (2004) *in* Ecological Genetics

Generating gene trees was once much more laborious; however, reliance on morphological and crude genetic techniques for constructing phylogenies ended in the mid-1980s with the invention of the polymerase chain reaction (PCR) (Mullis *et al.* 1986, Mullis and Faloona 1987). Kary Mullis won the Nobel prize for his 1985 achievement, and there was a veritable explosion of genetic information—especially sequence information—available to phylogeneticists when the technique became automated several years later (Smithsonian videohistory collection 1992).

These advances in genetic technology paralleled the birth of the field of "phylogeography", a term that John Avise first coined in 1987 (Avise *et al.* 1987). As Avise *et al.* (1987, 2000) point out, the idea that there are broad but general patterns of association between inter- or intraspecific phylogenetic variation and geography was by no means new, with many known studies investigating associations between restriction enzyme patterns and geographic distributions of species or populations that pre-dated the PCR revolution (the following examples are reviewed in Avise *et al.* 1987 and 2000: Endler 1977, Bermingham and Avise 1986, Lansman *et al.* 1983). Such studies were limited by the lack of high-resolution genetic information, so it was fortuitous that Avise's synthesis of phylogeography occurred just as PCR was becoming readily available to researchers in the field of ecological genetics.

Nuclear and mitochondrial gene sequencing eliminated the reliance on poorly-resolved restriction enzyme studies, and increasingly sophisticated models of nucleotide evolution have allowed greater insights into the evolutionary processes shaping the variation we observe in genealogies constructed on many scales, from intraspecific studies, such as the one this thesis focuses on, to interspecific studies spanning a potentially enormous range of taxa (Freeman and Herron 2001, Page and Holmes 2002).

Despite the leaps and bounds that have been made in the field of phylogeography over the last two decades, there is still a great deal of confusion and disagreement about what approaches are best for elucidating phylogeographic histories of species. This debate has evolved as the discipline has advanced, and I have found that my own views have evolved in a similar fashion, albeit rather more quickly.

As such, Lowe *et al.*'s (2004) statement starting this section represents a fundamentally flawed view, and it is a view that characterizes phylogenetic and phylogeographic studies with less and less frequency as time goes on, given the development of alternative approaches. The central issue is that this approach to phylogeography allows for studies in which there is no initial hypothesis, and *post hoc* hypotheses are created after genealogical data is generated and observed. There may be circumstances in which such a phylogeographic approach should be taken, for example, in biodiversity assays, but ultimately, research investigating associations between genetic variation and the geographic distribution of taxa being studied should be hypothesis-driven. A hypothesis, for example one based on observed morphological differences or clines, or one based on observed geographic barriers to gene flow, should always precede the collection of genetic data through sequencing or DNA fingerprinting. Certainly subsequent investigations via summary statistics can be based on observed genetic patterns, but even the collection of summary statistics should be underpinned by refutable hypotheses at some level.

There is an abundance of tree-building methods that can take into account complex models of sequence divergence and different methods of estimating genetic relationships between taxa, but they provide little in the way of hypothesis testing other than to give bootstrap or jackknife levels of confidence for a given model of divergence between taxa. Other approaches can deal with this dilemma, however. Statistical phylogeography encompasses a variety of techniques for testing *a priori* hypotheses about evolutionary histories that cause the phylogeographic patterns that Avise (1987) categorized, and its main advantage is the ability to

generate statistical estimates, and thus a degree of confidence, for those evolutionary histories, rather than relying on *post hoc* explanations (Knowles and Maddison 2002). Many of these techniques rely on creating and testing null and alternative models of evolutionary history (Hey and Machado 2003), among which one of the common approaches is the coalescent (Rosenberg and Nordborg 2002, Knowles 2001, Nichols 2001). An advantage of the coalescent technique is that it explicitly acknowledges the fact that gene genealogies may not reflect species histories (a particular problem with Lowe *et al.*'s (2004) approach to phylogeography), and in fact uses this disjunction between gene and species histories as the central component of statistical tests for hypothetical evolutionary histories (Maddison 1997, Rosenburg and Nordborg 2002).

Other techniques include Templeton's nested clade analysis (NCA), which very broadly speaking provides a more accurate analysis of genetic variation within a geographical framework (Templeton 1998, 2004). Methods for testing phylogeographic hypotheses also include the use of geographical information systems (GIS) to investigate complex patterns of geographically distributed genetic variation (eg. Kidd and Ritchie 2001, Ritchie *et al.* 2001), partial Mantel tests (eg. Ritchie *et al.* 2001, for discussion of their efficacy see Raufaste and Rousset 2001, Castellano and Balletto 2002, Rousset 2002), mismatch distributions (Avise 2000 and references therein) and network analyses (Bandelt *et al.* 1999, eg. Rocha *et al.* 2005). Many other simple analytical techniques like analysis of molecular variance (AMOVA) can be used to test *a priori* hypotheses, and for example have been employed recently by Knowles and Richards (2005) to distinguish genetic variation in a montane grasshopper that was due to early separation into glacial refugia from genetic variation due to more recent post-glacial recolonizations. The distinguishing feature of these studies is that the authors structure their analyses around initial hypotheses with testable predictions, as opposed to first generating data and then testing the patterns observed for significance.

The phylogeographic components of this PhD thesis make use of most of these, plus additional analytical techniques to test *a priori* hypotheses about Mormon crickets. Indeed, there is only one phylogenetic tree included in the entire thesis, and its input into the conclusions drawn is minor in comparison to the other analyses we performed on our sequence data. Perhaps some day in the not-distant future, phylogenetic trees will be superfluous, replaced instead by other more appropriate methods for visualizing and testing hypotheses about the manner in which genetic variation is spatially and temporally distributed.

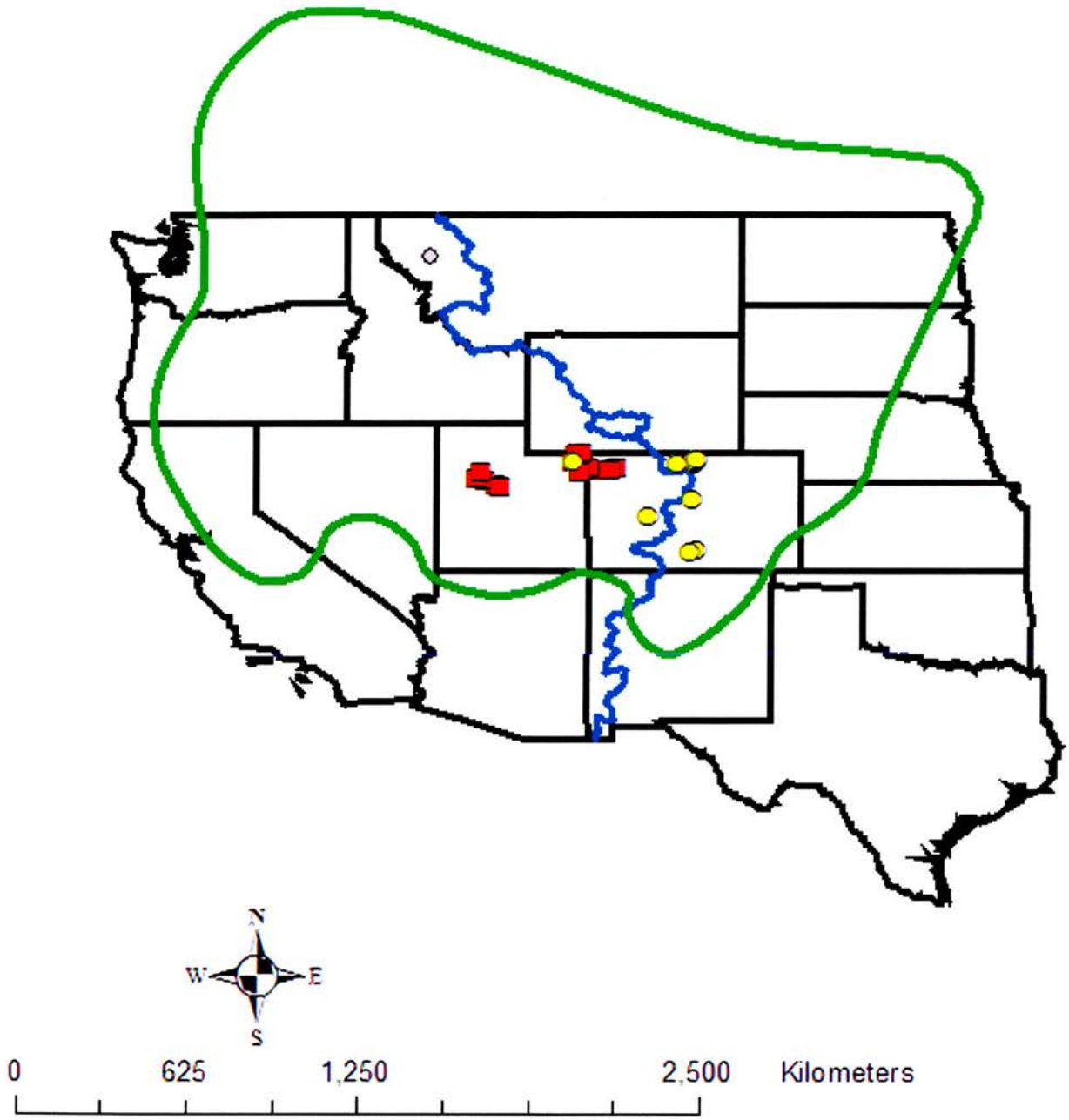


Figure 1: Range map of *Anabrus simplex* (adapted from Wakeland 1959). The approximate distribution of Mormon crickets is indicated by the green line, and the continental divide is in blue. Gregarious and solitary populations sampled for my thesis projects are represented by red squares and yellow circles, respectively. The intermediate-phase population from Montana is indicated by the grey circle.

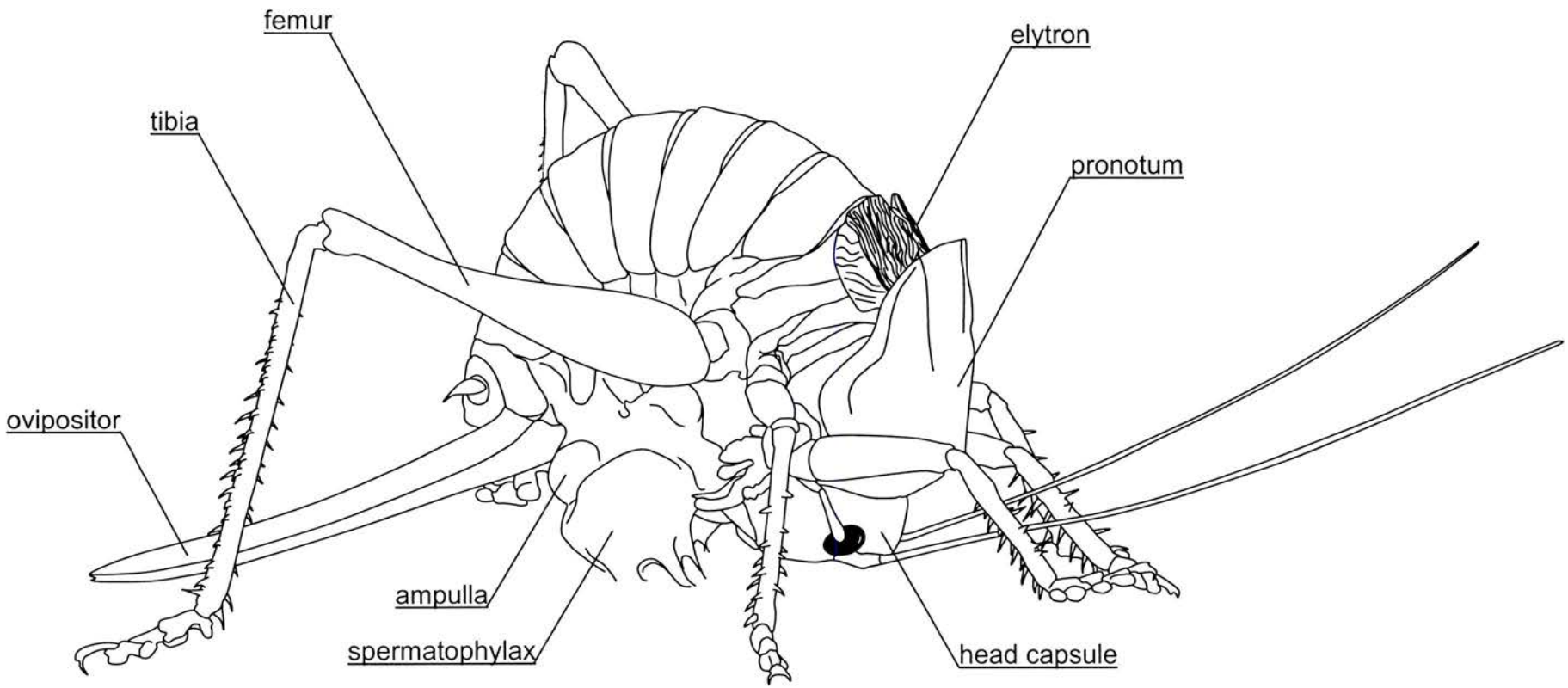


Figure 2: Female Mormon cricket eating spermatophore, which consists of a proteinaceous spermatophylax plus two sperm-containing ampullae.

Figure 3: Solitary female Mormon cricket showing typical cryptic colouration.

Figure 4: Kelly Flats site in Poudre Canyon, Colorado. This montane meadow is typical of solitary habitats.

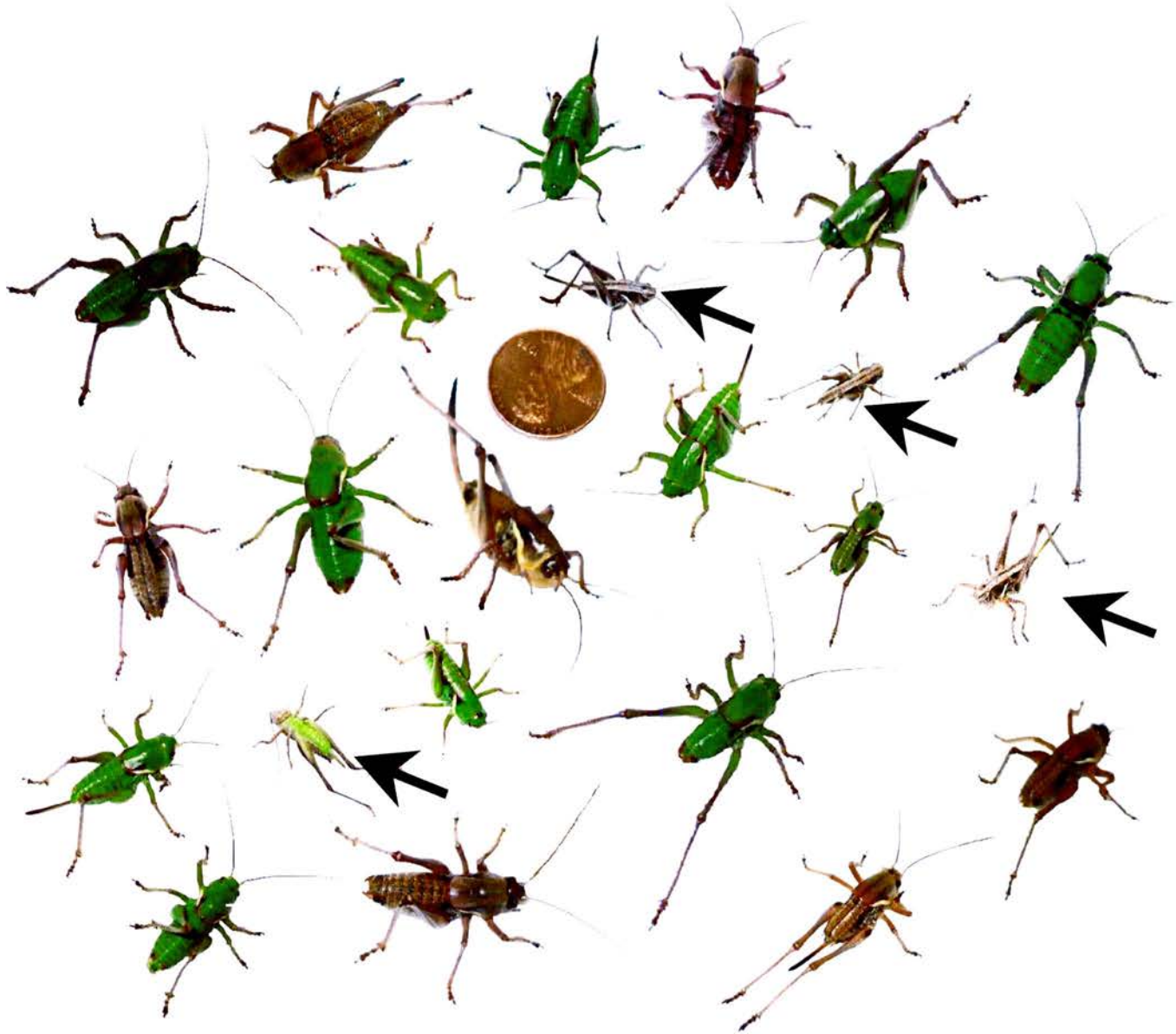


Figure 5: Variability in cryptic solitary phenotypes collected from Jack's Gulch in the Poudre Canyon, Colorado. Several *Steiroxys trilineatus*, another Tettigoniid, are indicated by arrows.

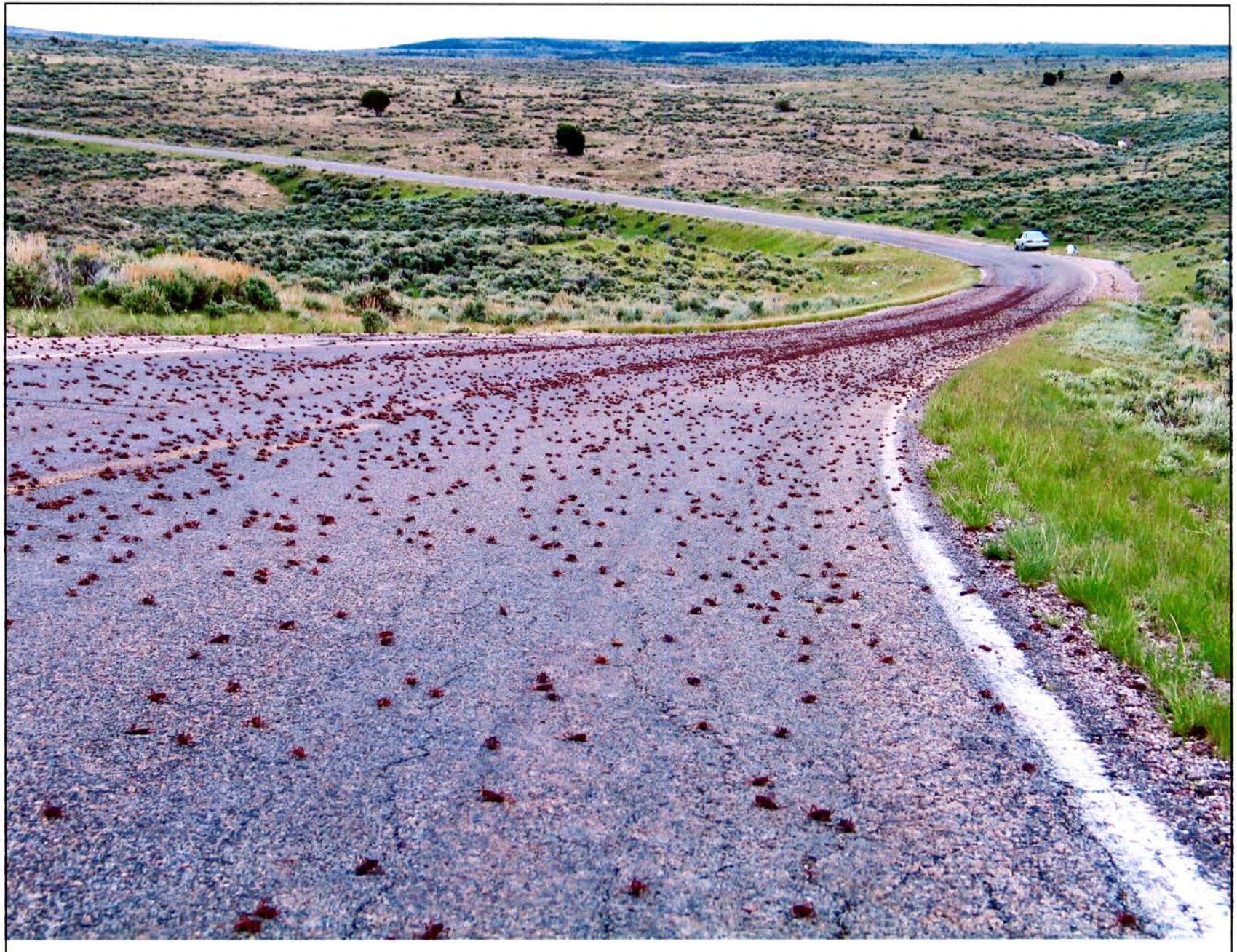


Figure 6: A gregarious band of Mormon crickets near Dinosaur, Colorado.

Figure 7: Gregarious Mormon crickets are aposematically coloured, and may sequester distasteful secondary compounds from sagebrush.

Figure 8: The accumulation of smashed gregarious Mormon crickets can cause dangerous road conditions.

ARE SOLITARY AND GREGARIOUS MORMON CRICKETS (*ANABRUS SIMPLEX*, ORTHOPTERA, TETTIGONIIDAE) GENETICALLY DISTINCT?[1]

NW Bailey, DT Gwynne, MG Ritchie

Abstract

Phase polyphenisms are usually thought to reflect plastic responses of species, independent of genetic differences; however, phase differences could correlate with genetic differentiation for various reasons. Mormon crickets appear to occur in two phases that differ in morphology and behaviour. Solitary individuals are cryptic and sedentary whereas gregarious individuals form bands, migrate, and are aposematically coloured. These traits have been thought to be phenotypically plastic and induced by environmental conditions. However, there has been no previous investigation of the extent of genetic differences between solitary and gregarious populations of this widespread North American species. We sequenced two mitochondrial genes, COII and COIII, in samples of Mormon crickets from gregarious populations west of the continental divide and solitary mountain populations primarily east of the divide. Sequencing revealed two genetically distinct clades that broadly correspond with the solitary eastern populations and the mainly gregarious western populations. We used coalescent modeling to test the hypothesis that the species consists of two deep genetic clades, as opposed to a series of equally distinct populations. Results allowed us to reject the null hypothesis that a radiation independent of phase produced these clades, and molecular clock estimates indicate the time of divergence to be approximately 2 million years ago. This work establishes that the solitary populations found in the mountains on the eastern slope are part of a clade that is

[1] **Bailey NW, Gwynne DT, Ritchie MG** (2005). Are solitary and gregarious Mormon crickets (*Anabrus simplex*, Orthoptera, Tettigoniidae) genetically distinct? *Heredity* **95**:166-173.

genetically distinct from the western populations, which are primarily gregarious, and the implications of this apparent correlation between phase and genetic differentiation are discussed.

Introduction

Phenotypically plastic traits that fall into discrete classes but do not reflect simple genetic polymorphism are often referred to as phase polyphenisms (Shapiro 1976). Solitary and gregarious phase polyphenisms are well known in swarming locusts (Orthoptera: suborder Caelifera), but have also been suggested in certain species of flightless bush crickets (katydids: Tettigoniidae) that can reach extraordinarily high population densities and migrate in bands (Cowan 1990, Gwynne 2001). Phase polyphenism can arise in response to differing cues during development such as resource availability or density (Evans and Wheeler 2001). In the desert locust *Schistocerca gregaria*, for example, population density mediates phenotypically plastic changes in juveniles (Sword and Simpson 2000, Simpson *et al.* 2001, Sword 2002). Under high density regimes, the locusts develop bright colouration patterns and form gregarious swarms. Such density-dependent phase polyphenisms may have evolved as adaptations to avoid predation pressures in desert locusts, with gregarisation enhancing the beneficial effects of aposematic colouration (Sword and Simpson 2002).

Selection on the extent to which phenotypic variation is determined by environmental cues can give rise to genetic differentiation between populations. Although plastic traits themselves may reflect no underlying genetic differences, the degree of plasticity is genetically determined, can vary, and is therefore subject to selection. When selection favours increased plasticity, plastic traits show more variable responses to their environmental cues. Phenotypic variation can thereby influence genetic variation through genetic assimilation in a series of gradual shifts in the trait optima of plasticity (Waddington 1953, Pál and Miklós 1999). Recent research has primarily focused on the environmental cues that induce phase polyphenisms in insects such as desert locusts; however, it is also important to study the genetic structure of such populations because of the potential influence that environmentally induced phase polyphenisms can have on genetic divergence.

Alternatively, phase polyphenisms could be genetically distinct if they result from or correlate with different evolutionary histories reflecting selection on traits, rather than selection

for or against increased plasticity of a particular trait. For example, many insects show a dimorphism resulting from a trade-off between flight and reproduction (Dingle 1985). The grasshopper *Chorthippus parallelus* is normally brachypterous but occasional macropterous forms are found. Two subspecies of this grasshopper differ substantially in how readily macropterous forms appear (Ritchie *et al.* 1987), perhaps as a result of ecological differences in their evolutionary history. Hence there will be a statistical association between morph and genetic differentiation which reflects history rather than a more direct association.

Mormon crickets (*Anabrus simplex*, subfamily Tettigoniinae) have a large geographic range covering nearly the whole of the western United States (Gurney 1939). The species appears to display two distinct phases (Cowan 1990) that have been assumed to be phenotypically plastic (e.g., Gwynne 1984), but any potential genetic differentiation is unknown. Solitary individuals occur at relatively low densities and are cryptically coloured. They commonly inhabit mountain meadows and have been studied mainly in canyons on the eastern slope of the Rocky Mountains in Colorado (Ueckert and Hansen 1970, Gwynne 1984, Lorch and Gwynne 2000). Gregarious populations occur in various habitats in western North America and are larger and darker in colour than solitary individuals on the eastern slope (Gwynne 1984). Gregarious Mormon crickets undergo destructive mass migrations and are a major crop pest that can cause tens of millions of dollars in damage in one season (Dunkle 2001). There is also evidence of life history differences between solitary and gregarious populations (Bailey and Gwynne pers. obs. 2004).

The phases of Mormon crickets also differ in mating behaviour. Gregarious females observed in north western Colorado compete aggressively for matings whereas males are choosy of mates. In contrast solitary crickets on the eastern slope of the Rockies do not show this role-reversal in behaviour (Gwynne 1981, 1984). Field experiments (Gwynne 1993) revealed that at high densities hungry gregarious females are sexually competitive in order to obtain a nuptial gift, the proteinaceous spermatophylax that forms part of tettigoniid males' spermatophores (Gwynne 2001).

These studies, in line with taxonomic work (Gurney 1939), assumed that solitary and gregarious Mormon crickets throughout the range comprise a single widespread species. However, distinct differences in size, colouration, behaviour and possibly life history, as well as the fact that solitary and gregarious populations in Utah and Colorado are usually separated by

the North American continental divide, raises the possibility that the two forms have distinct evolutionary histories. Although solitary Mormon crickets can be found at high altitudes, the continental divide may be a significant impediment to gene flow in this flightless species, particularly for solitary individuals because they move less than gregarious individuals (Lorch and Gwynne 2000). Are there genetic differences between gregarious and solitary populations of *A. simplex*, or are the distinct differences in phenotype simply a phase polyphenism due to phenotypic plasticity? If there is genetic differentiation, how great is it?

To answer these questions, we sequenced segments of two mitochondrial genes (COII and COIII) in Mormon crickets. These genes are maternally inherited and non-recombining, and have been useful for testing genetic differentiation in other Orthopteran species in which primers have been developed (Simon *et al.* 1994). We sampled individuals from gregarious populations close to previous study sites in Utah and north western Colorado and several solitary populations from the eastern slope of the Rockies. However, we also included two solitary populations found west of the continental divide, one of which was about 50 km from gregarious populations. We tested the null hypotheses that a) the gregarious and solitary populations in previous study sites show no genetic differentiation, and b) genetic differences between populations within each phase are of equal magnitude. Phylogenetic analyses enabled us to reject both hypotheses. Moreover, the analysis uncovered two very distinct clades of Mormon crickets from the eastern and western slopes of the Rockies that broadly correspond to solitary and gregarious phase.

The population structure in Mormon crickets was further investigated using a non-phylogenetic method of inference. Coalescent modeling is a hypothesis-driven technique that can be used to test alternative evolutionary histories in organisms. Lineages are sampled backwards through a constructed genealogy of the populations in question, and hypotheses are tested by assessing whether the rate at which lineages coalesce deviates significantly from the rate predicted by evolutionary models (Rosenberg and Nordborg 2002). This technique has been used to address similar questions in several studies, including one of the montane grasshopper *Melanoplus oregonensis* found in the Rocky Mountains (Knowles 2001). Coalescent modeling can overcome problems with phylogeographic methods, mainly that the gene history derived from molecular markers may not match the organismal history, which is more likely to occur when examining closely related species or populations (Maddison 1997, Rosenberg and

Nordborg 2002, Hey and Machado 2003). It is therefore a superior tool for analysing population structure: we tested the external hypothesis that the phylogenetic pattern observed is consistent with a deep subdivision broadly coincident with the solitary and gregarious forms, for example one that may have occurred during repeated glacial cycles, as opposed to population diversification from a common ancestor independent of phase.

Materials and Methods

Sampling

Samples were collected in 2001 from several individuals in each of ten locations in Utah and Colorado, with seven solitary and three gregarious populations represented (Figure 1). One of the solitary populations was collected from Little Brush Creek, Utah, which was 47 km from the gregarious Echo Park and Dinosaur Park populations. The five populations collected on the eastern side of the continental divide were solitary, whereas three of the five populations west of the divide were gregarious.

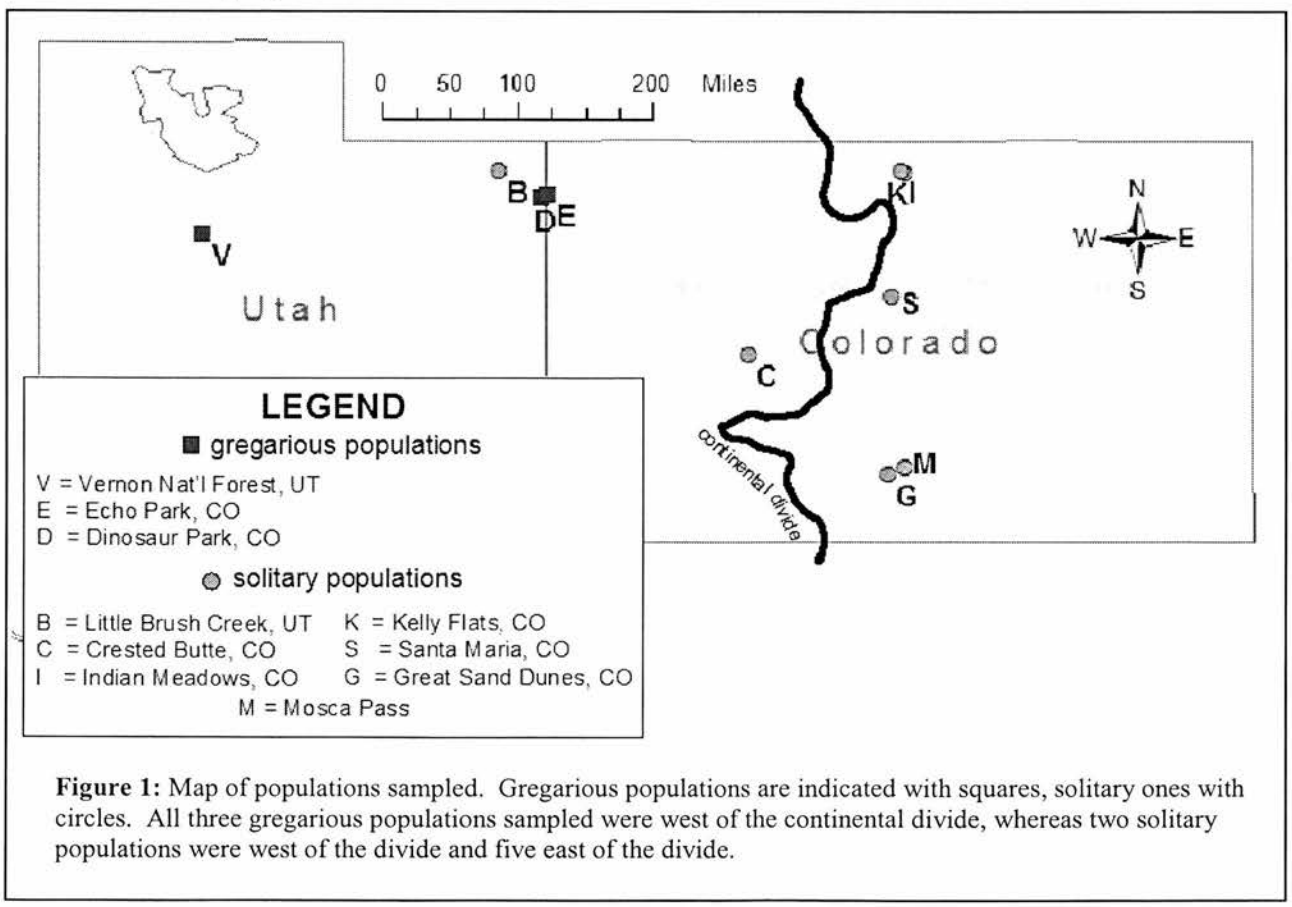


Figure 1: Map of populations sampled. Gregarious populations are indicated with squares, solitary ones with circles. All three gregarious populations sampled were west of the continental divide, whereas two solitary populations were west of the divide and five east of the divide.

DNA extraction, amplification and sequencing

DNA was extracted following the PureGene protocol (Gentra Systems, Minneapolis, Minn.), and quantified against known concentration standards. Forward and reverse primers C2-J-3279 and TD-N-3862, respectively, were used to amplify a 440 bp region of cytochrome oxidase II mitochondrial gene (COII), and forward and reverse primers C3-J-5014 and C3-N-5460, respectively, were used to amplify a 410 bp region of cytochrome oxidase III mitochondrial gene (COIII). Primer sequences were from Simon *et al.* (1994). PCR reactions were carried out in 50 μL volumes and contained: 0.2 mM each dNTP, 0.20 μM each primer, 1.5 μL 10X NH_4 reaction buffer, 1.5 mM $MgCl_2$, 0.33 μL Biotaq DNA polymerase, and 10-300 ng DNA template. The PCR profile for all reactions was: (1) 5 min at 92°C initial denaturation, (2) 10 sec at 92°C denaturation, (3) 15 sec at the annealing temperature (47°C for COII and 46°C for COIII), (4) 1 min at 72°C extension, (5) 39 repeats of steps 2 through 4, and (6) 10 min at 72°C final extension. PCR products were checked on 1.5 % agarose gels and sequenced using a Beckman-Coulter automated sequencer. Raw sequences were edited and aligned using Chromas 2.12 (Technelysium, 2001) and ClustalX 1.81 (Thompson *et al.* 1997), respectively. Forward and reverse sequences were produced for each individual and aligned to reduce the likelihood of sequencing errors. Generally there was little discrepancy, although sequences were re-run if forwards and reverses did not correspond. All sequences corresponded in the end.

Phylogeographic analysis

We tried but were unable to sequence both COII and COIII in various outgroup species because of difficulties with PCR amplification. We have therefore included two tettigoniid species as outgroups: *Ephippiger ephippiger* (subfamily Bradyporinae) and *Kawanaphila gidya* (Zaprochilinae). Outgroups in the analysis were sequenced for either COII or COIII.

We used Modeltest 3.06 (Posada and Krandall 2001), in conjunction with PAUP* 4.0 (Swofford 1998), to assess which model of substitution was most suitable for these sequences, and the Tamura-Nei model with a gamma distribution shape parameter of 0.0937 was most appropriate. Instead of analysing sequences from both genes separately, we used the partition homogeneity test in PAUP* to test if the phylogenetic signals from the two genes were

congruent. They were, so the two sequences were concatenated and analysed together. To align the outgroups with the concatenated sequences we simply coded the absent gene as missing data. A maximum likelihood tree for the concatenated sequences was constructed in PAUP* (Swofford 1998) using 20 replicates. A neighbour-joining tree with bootstrap values generated using 500 replicates was also produced in MEGA v.2.1 (Kumar *et al.* 2001) and compared with the maximum likelihood tree.

We divided our subsequent analyses into two categories: the first set was to test external hypotheses about whether genetic differentiation in our populations of Mormon crickets is coincident with phase, so these analyses were carried out independent of the structure found in the mtDNA phylogeny. The second set was carried out to generate summary statistics on the genetic clades uncovered in the phylogeny. These analyses therefore specifically addressed the structure found in the mtDNA phylogeny and were carried out independent of phase.

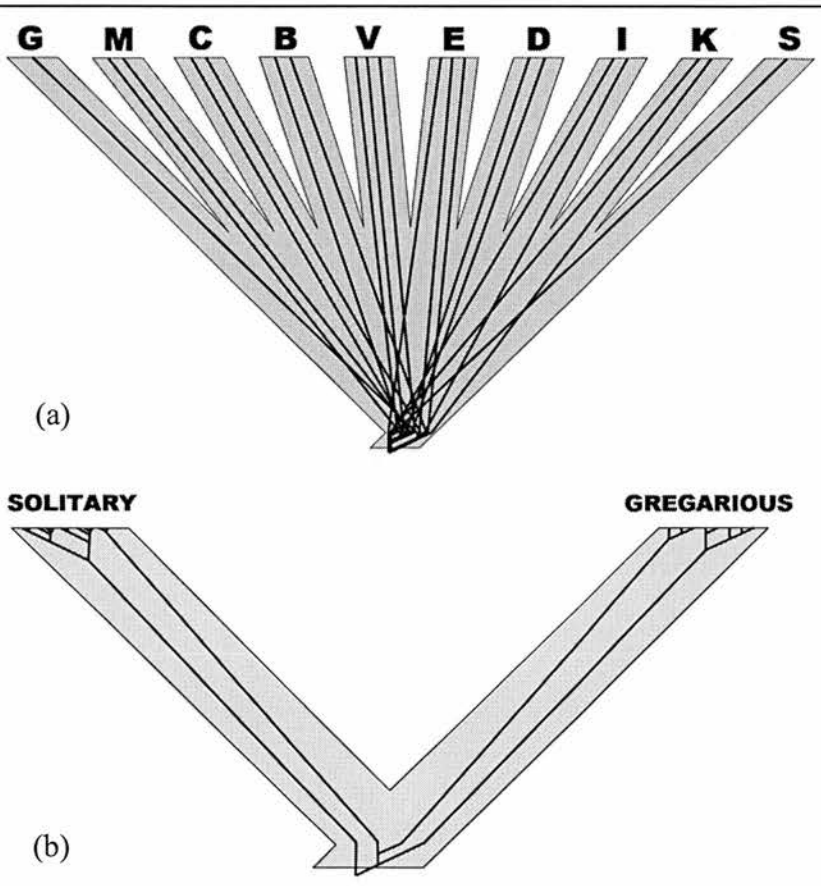


Figure 2: (a) Diagram of null population model. Lines within the tree represent individual gene trees, or lineages, and each branch represents one population (labels correspond to Figure 1). This star-shaped tree models rapid fragmentation of an ancestor, with populations diverging independent of phase. We simulated 1000 gene trees within this population tree using neutral coalescence. **(b)** Diagram of "deep clade" alternative population model in which simulated and empirical gene trees were contained. The two branches represent a deep phylogeographic split corresponding with phase.

Coalescent analysis

Coalescent modelling can be used to evaluate the level of discordance between population histories and gene trees. One measure of this is Slatkin's *s*, which measures the number of additional parsimony steps in a given tree that are needed for the

gene tree to correspond to the population history—denoted as a population tree—in which it is contained (Slatkin and Maddison 1989, Knowles 2001). Therefore, instances of incomplete lineage sorting, called "deep coalescences", cause greater discord between gene and population trees, which produces a higher *s* value.

Our null hypothesis was that the population structure observed in Mormon crickets results from population fragmentation independent of the phases, which would predict no significant genetic differentiation between populations grouped by phase. The alternative hypothesis is that the two forms reflect distinct evolutionary histories resulting from an older division within the species, for example due to glacial periods in the Pleistocene.

We used the program Mesquite v.1.0 (Maddison and Maddison 2004) to test these two evolutionary histories. First, we created a star-shaped population tree with 10 branches that reflects the evolutionary history under the null hypothesis—a fragmentation independent of phase (Figure 2a). Each branch represents one population that we sampled. We then simulated 1000 gene trees within this population tree using neutral coalescence. To test the alternative hypothesis, we contained the simulated gene trees within a V-shaped population tree that had two deep branches representing a split between solitary and gregarious populations—the 'deep clade' tree (Figure 2b). We used Slatkin's *s* to measure the discordance between the gene trees simulated in the null population tree and the alternative deep clade tree in which they were contained. Finally, we contained our reconstructed empirical gene tree produced in PAUP* within the deep clade tree and measured the discordance. If the *s* value of our reconstructed tree falls below 95% of the distribution of simulated gene tree *s* values, we can reject the null model in favour of the deep clade model. It should be emphasised that we did not test the relative timing of population divergence under these scenarios; here we concentrate on only testing the particular hypothesis that there is a single large genetic subdivision broadly coincident with the phases of this species.

Separately, an overall nested AMOVA (analysis of molecular variance) (populations nested within phase) was performed in Arlequin ver. 2.000 (Schneider *et al.* 2000) to assess the proportion of genetic variation that was distributed between the phases and among populations within phases, which might be expected to differ given the differences in movement.

Summary statistics

Two analyses were performed to generate summary statistics on the two clades uncovered by our phylogenetic analysis. We used the DNADIST v. 3.6 executable in PHYLIP (Felsenstein 2004) to determine pairwise distance measures using a Jukes-Cantor correction between individuals in the two clades, from which we then calculated the mean percentage of sequence divergence between the clades following the method of Lunt *et al.* (1998). We extrapolated an approximate time since divergence from this information. To gauge differences in population structure within the two clades, a separate AMOVA was performed on each to compute the percentage of among vs. within population variation, allowing us to assess whether differentiation among populations was greater within either clade. AMOVAs were perfomed in Arlequin ver. 2.000 (Schneider *et al.* 2000).

Results

Phylogeographic analysis

The concatenated sequences from 37 individuals yielded an 850 bp fragment with 79 parsimony-informative characters and 20 discrete haplotypes. The topology of the maximum likelihood tree was similar to that of a neighbour-joining tree constructed in MEGA 2.1 (Kumar *et al.* 2001) using the Kimura-Nei model.

There are two noteworthy patterns in the tree, shown in Figure 3. First, the samples fall out into two discrete clades that broadly correspond with phase: a central and eastern Colorado mountains clade of only solitary populations ("eastern" clade) and a clade of populations from west of the Colorado Rockies that is mainly comprised of gregarious populations ("western" clade). The Little Brush Creek solitary population (coded "B") is the only solitary population that groups with the gregarious ones in the western clade. Despite this exception, an overall nested AMOVA indicated significant genetic differentiation between solitary and gregarious populations with 10.57% of the genetic variation distributed between the phases ($p = 0.028 \pm 0.005$). Second, compared to the western clade there is more genetic structure in the eastern clade, where many populations form monophyletic groups and are supported by high

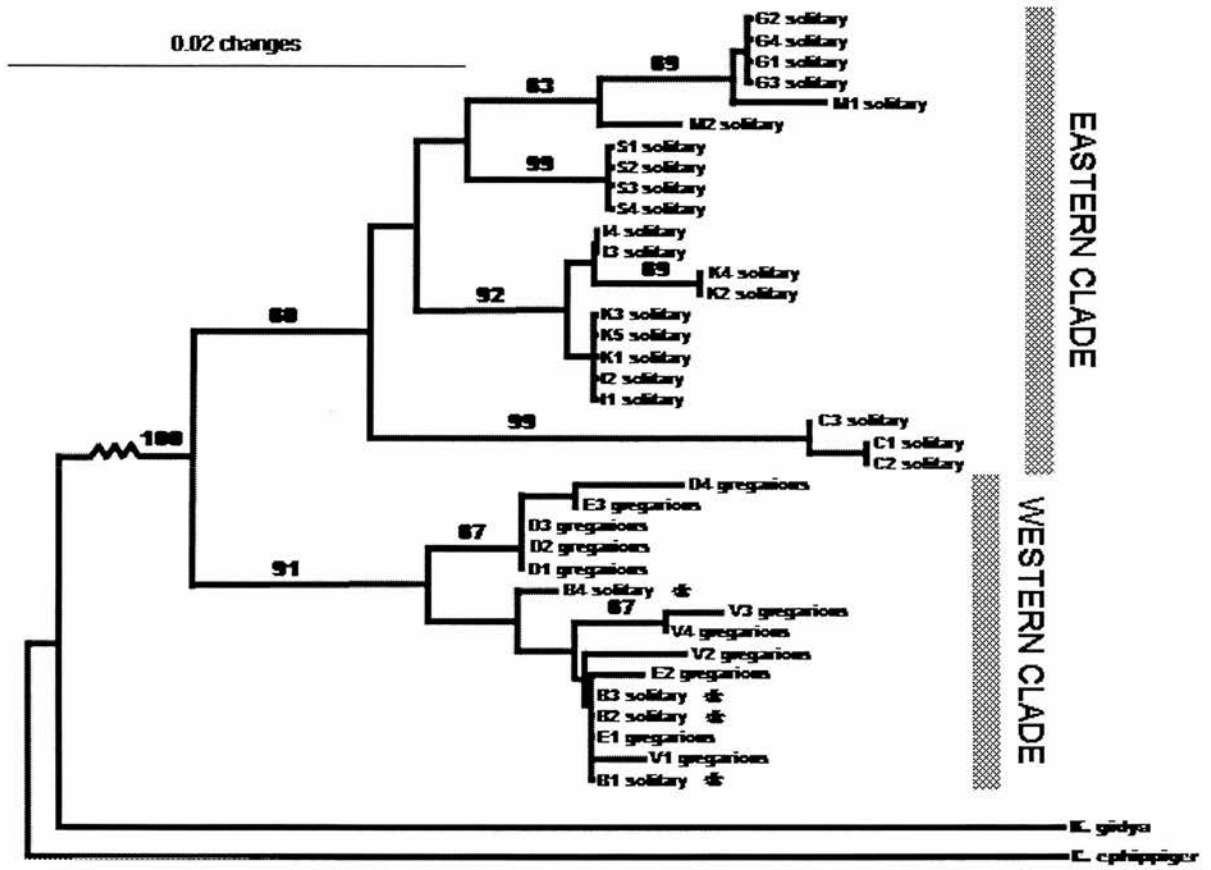


Figure 3: Tamura-Nei NJ tree using COII and COIII sequences combined (MEGA v.2.1). Bootstrap values over 80% are indicated. Sample labels are as in Figure 1, and western and eastern clades are indicated. Little Brush Creek solitary samples falling in the western clade with gregarious populations are labeled with asterisks (*).

bootstrap values. Branch lengths in the western clade are shorter, populations generally do not form monophyletic groups, and bootstrap support is low.

Coalescent analysis

Figure 4 shows the *s* values produced when we took 1000 gene trees simulated by neutral coalescence in our star-shaped null population history model and contained them within the V-shaped deep clade model. The *s* value of our reconstructed empirical tree when contained within the alternative model was 3, indicated by the dashed line in the histogram. It falls below 95% of the distribution of simulated gene trees, so our empirical tree rejects the null hypothesis in favour of the alternative deep-clade model.

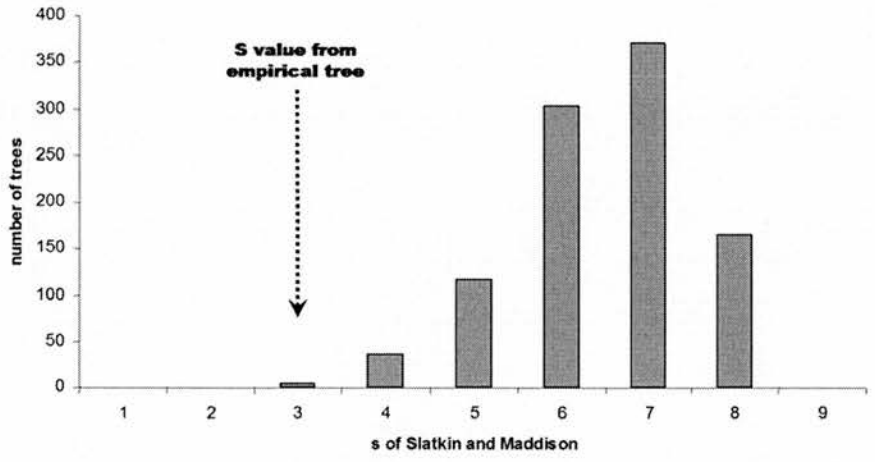


Figure 4: Histogram of *s*-values generated when trees constructed using neutral coalescence in the null population model were contained within the alternative deep clade model. The *s*-value generated when the mtDNA gene tree for our Mormon cricket populations was contained within the alternative deep clade model is indicated by the dashed line.

Divergence of geographic clades

The mean percentage of sequence divergence between individuals from different clades was 3.98, s.d. ± 0.43%. Given an approximate rate of sequence divergence of 2% per million years (Brown *et al.* 1979, Lunt *et al.* 1998, Grant and Miles 2002), divergence time for these clades is approximately 2 million years ago.

Separate AMOVAs for each clade indicate that there is more differentiation within the eastern clade, with 80.86% of genetic variation attributable to between population differences,

whereas in the western clade only 39.07% of the variation is between populations. Conversely, within population variation is lower in the eastern clade (19.14%) than in the western clade (60.93%).

Discussion

The broad genetic division between western (mainly gregarious) and eastern (solitary) populations of *Anabrus simplex* is an unexpected result given the previous assumption that gregariousness in this species is a phenotypically plastic trait (Gwynne 1993). Our results show that there is a significant genetic division within *Anabrus simplex*; solitary and gregarious populations on the east and west slope of the Rockies, respectively, form two discrete clades. We raised two contrasting hypotheses regarding the nature of these clades and the phase of the populations comprising them. In one, populations differ without respect to phase whereas in the other, the phases represent or coincide with a deep phylogeographic split within the species. Under the former scenario, we would have expected a phylogenetic analysis to indicate either complete panmixis or paraphyletic groups for each population over the whole species. However, our analyses reject the former scenario in favour of the latter; in the populations that we sampled there is significant genetic differentiation between the phases and the phylogeographic history more strongly supports the second hypothesis. The division is not perfect, however, because the solitary Little Brush Creek population—sampled just 50 km from gregarious populations in the west—falls in the clade with all gregarious populations. Given that the data overall support a complex history behind the phases, incorporating plasticity, what does the genetic division suggest about the evolutionary history of the two forms?

Early origin of the division

Based on the sequence divergence between the eastern and western clade, we estimate that the major clades diverged around 2 million years ago. Having diverged before the last ice age, they must have quite distinct evolutionary histories during which genetic differences have accumulated. Such differences may influence a range of traits, including the environmentally induced threshold influencing the switch point between alternative phases. In general, such

switch points between alternative morphologies are known to be genetically determined and to evolve, sometimes rapidly (e.g. Tomkins and Brown 2004). An alternative to our favoured hypothesis of differing thresholds between two genetically distinct forms is that the threshold remains similar but environmental conditions are "fixed" between eastern and western populations. In addition to the genetic divergence, these clades differ in morphology and life history characteristics.

There is no formal agreed definition for subspecies (like species), but a working hypothesis is that subspecies should differ at numerous concordant traits yet lack reproductive isolation (Avise & Ball 1990). The continental divide is a site of numerous hybrid zones between subspecies (though see Swenson and Howard 2004). Our analyses indicate that the two clades within Mormon crickets have probably been evolving independently for around 2 million years, which is consistent with other studies of subspecific variation (eg., Hewitt 1996, Lunt *et al.* 1998) and must have occurred over several glacial cycles. Indeed, many sister species of North American birds show less mtDNA divergence (Lovette and Bermingham 1999, Lovette 2005). During periods of allopatry, genetic differences between eastern and western Mormon crickets could have accumulated either by genetic drift or via selection for variation in response to differing environmental cues. Further studies of Mormon crickets will be necessary to fully assess the level of divergence between these forms and over what geographic scale the division remains correlated with phase.

In our samples, there is one interesting and important exception to the coincidence between phases and the deepest clade: the solitary samples from Little Brush Creek, UT cluster in the western clade. There are several possible explanations for this. Despite the general coincidence, exceptions are expected given the importance of environmental condition in any one year in generating a phase switch. This population might experience unusual local conditions, or have a particularly high switch point for becoming gregarious. Based on food-manipulation experiments, Gwynne (1993) concluded that courtship role reversal, seen in gregarious populations, is a result of low food availability due to high population density. Further support for the idea that food quality causes a switch in phase from solitary to gregarious comes from other studies of Mormon crickets and the desert locust *Schistocerca gregaria* (Gwynne 1984, Simpson *et al.* 2001).

Other possibilities include migration and or hybridisation. Gregarious bands of Mormon crickets originating in the west have been observed within one kilometer of the Little Brush Creek site (Bailey pers. obs. 2004) and if gregarious individuals strayed from their band and colonized the area, subsequent generations may have matured in conditions that were not conducive to gregarious band formation, eg. low density, high nutritional resources etc, and become solitary. This could explain why there is virtually no genetic difference between the Little Brush Creek solitary population and all the gregarious populations. Alternatively, there may be a high level of gene flow into the Little Brush Creek population because gregarious Mormon crickets are by their nature very mobile. If no barriers to reproductive isolation exist between the two phases, bands of gregarious individuals from nearby may have interbred with Little Brush Creek crickets in the past. Although solitary Mormon crickets at Little Brush Creek reach reproductive maturity four to six weeks later than the gregarious ones nearby (Bailey and Gwynne, pers. obs., 2004), once they are adults they may be capable of mating with migratory gregarious individuals later in the season.

Differences in population structure between eastern and western clades

Separate AMOVA analyses indicate that populations in the western clade show much less genetic structure than populations in the eastern clade. This is not surprising because gregarious Mormon crickets form bands that travel far and wide, sometimes marching further than one kilometer per day (Lorch and Gwynne 2000). There are even accounts of separate bands intersecting one another (MacVean 1987), and these band movements will provide opportunities for interpopulation matings and gene flow. Populations in the eastern clade, however, are much less likely to disperse because they inhabit small areas of meadow habitat within mountain canyons and do not move over very long distances (Lorch and Gwynne 2000) thereby decreasing the likelihood of gene flow between other solitary populations. Over historical timescales, these different migration rates may have had a homogenizing effect on the genetic structure of gregarious populations and may have facilitated differentiation amongst solitary populations.

Implications

The specific assumption in Gwynne's (1984) observational work showing different sex roles in Mormon crickets was that eastern Poudre Canyon and western Dinosaur National Monument populations were not genetically different. The mtDNA evidence presented here, however, challenges the implicit assumption that phase polyphenisms, and hence sex role-reversal, in those populations are solely plastic traits, in favour of the idea that phase broadly coincides with an ancient subdivision within the species. As with any example of phenotypic plasticity, exceptions will occur and the Little Brush Creek population provides an example. Further studies must concentrate on improving the geographic sampling to better assess the extent of coincidence between phase and genetic differentiation.

Even though the genetic differentiation seen in Mormon crickets is consistent with subspecies or even species status, such a conclusion should not be based simply on levels of genetic differentiation. What makes this system novel is that a suite of morphological, behavioural and life history traits are broadly coincident with the solitary/gregarious phase difference in addition to the genetic differentiation. If we are correct in considering the differentiation between clades similar to that between subspecies, it will be worth re-examining other trait differentiation between the clades, especially including behaviours and other traits with the potential to influence reproductive isolation and gene flow between these forms.

Acknowledgements

Thanks to P. Lorch for help collecting crickets, L. Hockham for his valuable assistance in the early stages of molecular lab work, K. Vahed for providing outgroup samples, R. Hamill for encouragement during those long dark hours in the lab, and D. Kidd and L. Knowles for elucidating the coalescent. This work was funded by an Overseas Research Students Award Scheme and the University of St. Andrews.

DEVELOPMENT AND CHARACTERIZATION OF MICROSATELLITE LOCI IN MORMON CRICKETS (*ANABRUS SIMPLEX*, ORTHOPTERA, TETTIGONIIDAE)[2]

NW Bailey, LR Hockham, JA Graves, MG Ritchie

Abstract

Anabrus simplex is an economically significant crop pest in the western United States and is a model organism for studying the influence of sex role reversal on sperm allocation and utilization patterns and population genetics. We isolated seven polymorphic microsatellite loci in *Anabrus simplex*, and within population allele numbers ranged from 10 to 25. High polymorphism is not unusual for Orthopteran insects, although observed heterozygosities ranged from 0.24 to 0.91 and were lower than expected heterozygosities, suggesting null alleles. These microsatellites will greatly facilitate studies of post-copulatory reproductive isolation in nuptial gift-giving insects and historical phylogenetics in the Rocky Mountains.

Introduction, materials and methods, results and discussion

The range of Mormon crickets (*Anabrus simplex*, subfamily Tettigoniinae) covers nearly the entire western United States, and they occur in two phases that are assumed to be phenotypically plastic (Gwynne 1984). One phase, which occurs primarily on the eastern slope of the Rocky mountains, is sedentary and cryptically coloured, whereas the other phase occurs west of the Rockies and forms large bands that can migrate up to one kilometer per day (Lorch

[2] **Bailey NW, Hockham LR, Graves JA, Ritchie MG** (2005) Development and characterization of microsatellite loci in Mormon crickets (*Anabrus simplex*, Orthoptera, Tettigoniidae). *Molecular Ecology Notes* **5**:613-615.

and Gwynne 2000). These phases have been referred to as solitary and gregarious, respectively (though see Sword 2005). Like other Tettigoniids, male Mormon crickets donate a nuptial gift to females upon mating which consists of a large proteinaceous spermatophore. Mating roles are reversed in solitary and gregarious populations; females are choosy of males in the former whereas males are choosy of females in the latter due to differences in relative mating investment between the two forms (Gwynne 1981, 1993). The existence of large nuptial gifts and courtship role reversal provides fertile ground for studies of sperm competition, cryptic female choice, and polyandry in this unique species. Additionally, few studies have focused on the population genetics of Mormon crickets, and the only phylogeographic study to date has involved mtDNA (Bailey *et al.* 2005a). Nuclear genetic markers would enrich these studies; however, nuclear sequences in this species have yielded insufficient variation to allow any resolution (unpublished data). Microsatellites are a more useful tool because of their higher mutation rate and greater levels of polymorphism; therefore, we isolated several highly polymorphic microsatellite loci in Mormon crickets.

Genomic DNA was extracted from the hind femurs of 20 individuals from both solitary and gregarious populations in Colorado and Utah, respectively, using the PureGene protocol (Gentra Systems, Minneapolis, Minn.). This DNA was pooled and used to create an enriched library using the method of Hammond *et al.* (1998) in which CA and GA repeats were targeted using biotinylated CA and GA oligonucleotides.

Supercompetent *E. coli* cells (Stratagene) were transformed with the enriched library fragments which had been ligated to vectors. In total, 1810 clones were retrieved after having been grown and screened on amp+ and tet+ agar, and 34 positive clones were identified. One clone contained an insert that would not amplify using PCR, and the insert of another was too long to sequence, so we forward and reverse sequenced the remaining 32 using universal M13 primers on a Beckman-Coulter automated sequencer. Repeat motifs were identified in 22 of the sequences, and primers were designed for 18 loci that had adequate flanking regions. Of these, we were able to optimize 10 using variable PCR conditions. We were unable to optimize the remainder even after redesigning the primers. PCR reactions were carried out in 15 μL volumes and contained: 0.20 mM each dNTP, 0.30 μM each primer, 1.5 μL 10X NH_4 reaction buffer, between 1.0 and 2.0 mM $MgCl_2$, 0.10 μL Biotaq DNA polymerase (Bioline), and 10-300 ng DNA template. The PCR profile for all reactions was: (1) 2 min at 92°C initial denaturation, (2)

30 sec at 92°C denaturation, (3) 20 sec at the annealing temperature (see table 1), (4) 30 sec at 72°C extension, (5) 39 repeats of steps 2 through 4, and (6) 5 min at 72°C final extension.

Polymorphism was detectable on either ordinary agarose or Metaphor agarose gels (BioWhittaker Molecular Applications) in 7 of the 10 optimised loci, and the remaining 3 were monomorphic. Table 1 gives annealing temperatures and diversity information for the 10 optimised loci. We screened the 7 polymorhpic loci in populations of Mormon crickets of between 27 and 74 individuals. Some loci, for example 782, were extremely polymorphic, so we tested for a correlation across loci between sample size and number of alleles, which was not seen ($n = 7$, $r^2 = 0.050$, $p = 0.92$).

Microsatellites in Orthopteran species are known to be extremely polymorphic, but observed heterozygosities are frequently lower than expected heterozygosities (Hockham *et al.* 1999, Zhang *et al.* 2003) Loci 111, 129, and 146 in this study deviated significantly from Hardy-Weinberg equilibrium (respectively: $n = 29$, $p<<0.001$; $n = 26$, $p<<0.001$; $n = 74$, $p<<0.001$). Deviations from Hardy-Weinberg equilibrium may differ between populations, however, so even if heterozygosity is lower than expected in a given population, others may remain in Hardy-Weinburg equilibrium. We screened loci 111, 129, 383 and 782 in an initial test population, but due to the presence of null alleles at loci 111 and 129 we did not screen those two in larger populations. We used Genepop v3.4 (Raymond and Rousset 1995) to test for linkage between the remaining five loci (146, 383, 390, 782 and 1710), which we did screen in a larger population, and no pairs deviated from equilibrium. Using the same procedure, we tested for linkage between the four loci screened in the test population, and no pairs deviated from equilibrium. The low recovery rate of loci from libraries in this and other species suggests that in general Orthoptera may have low numbers of microsatellite loci.

Acknowledgements

This work was supported by the University of St. Andrews and an ORS Award to NWB and was carried out in the Molecular Ecology laboratory at the University of St. Andrews. Thanks to DTG for specimen collection and to RH for help in the lab.

Table 1: Description of optimized Mormon cricket microsatellite loci

Locus	Primer sequences 5' to 3'	Size Range	Repeat Structure	T_a (°C)	No. of alleles	*Ho*	H_E	GenBank accession no.
75	F AGGCAGCAATCCTTAGTGGA R AGAAGAAGCCCAGTGTGTTCA	300	$(CA)_3$	52	1	----	----	AY927781
111	F TTCATGAATGCGTTTTCAGC R ATTCCGATGGCACTGAGACT	173 - 255	$(CA)_8CG(CA)_5(CG)_9(CA)_3$	54	10	0.24	0.77	AY927773
129	F AGTGAGGCTTATCGTGGTCC R GGTTGAAGCTTTCCTTGTGC	222-390	$(GAAT)_6$(90bp)$(GA)_2(TA)_4(GATA)_2(TA)_2$	56	16	0.67	0.86	AY927780
146	F TTCAACTCCAAAGCAATTAAAATAA R GATTGATTGATTGATTGAACTCTAAA	265-455	$(AC)_{13}CG(AC)_{13}$	49	17	0.73	0.93	AY927782
383	F CACACTGTCGTAACACGGTC R AAGCAGCCATTGACTGATGA	190-222	$(CA)_{12}$	47	10	0.74	0.85	AY927776
390	F GTGGCACACAACGATAAACG R GAATGTTGACGTGACCATGTTT	212-280	$(CA)_{19}$	62	15	0.91	0.88	AY927778
782	F GGCAAGCAGTTATTTGCTCC R GTCCGCCATTCATAACGACT	83-193	$(CA)_{50}$	57	25	0.79	0.95	AY927779
1195	F TGGTCCGGGTTTGTTTAGTT R GCAAGCGTAAACATTGAGCA	154	$CAA(CA)_2CAA$	54	1	----	----	AY927777
1710	F ACGTTTCTCTGGGTTTGTGG R TGAGGGTGTGATTGATTCCA	147-189	$(GA)_{16}GG(GA)_3$	55	10	0.76	0.73	AY927774
1725	F CATTCTTTCTTCTGCGTACTAAAA R GATGGACAGACTGGGCACTT	238	$(CA)_{36}(TACA)_3TAC(GT)_5CAC(GAA)_2$	55	1	----	----	AY927775

Differences In Dispersal Behaviour Predict Population Genetic Structure In Mormon Crickets

NW Bailey, DT Gwynne, MG Ritchie

Abstract

The role of Pleistocene glaciations in promoting divergence has been widely studied, especially in the context of Remington's suture zones. Few of his original North American suture zones enjoy statistical support, but the Rocky Mountains provide an exception and have been implicated in deep intraspecific mtDNA divisions in several species. The aim of this study was to better understand how genetic population structure reflects recent divergence due to life history differences, superimposed on older, deeper divisions due to Pleistocene vicariant events. Mormon crickets exist in two phases, solitary and gregarious, that broadly correspond with a significant east-west genetic division within the species. This study tests several *a priori* hypotheses using mitochondrial and nuclear markers. First, our results reject the hypothesis that phase has had an influence on genetic divergence in favour of the hypothesis that the Rocky mountains were a barrier separating Mormon cricket populations into eastern and western refugia during Pleistocene glacial cycles, thereby causing a deep mtDNA division within the species. Second, isolation by distance differs significantly between eastern and western clades for both sets of markers, and a mismatch distribution analysis indicates historical population expansion in the western refugial population but not in the eastern one. Marked differences in dispersal ability have most likely influenced this more recent population genetic structure. The phylogeographic and demographic patterns in Mormon crickets correspond to those in other species, further supporting a broad, but generalized, pattern of divergence affecting species in the Rocky Mountain region during the Quaternary.

Introduction

Since Avise *et al.* (1987) first coined the term phylogeography, researchers have used molecular genetic techniques to investigate the influence of Pleistocene glaciations on the broad phylogeographic patterns we observe in extant species (reviewed in Hewitt 2004). Recent empirical studies have confirmed that Pleistocene glaciations are likely to have driven diversification in some North American species (eg. Knowles 2001, Ayoub and Riechert 2004, Weir and Schluter 2004, Nice *et al.* 2005), but this model is not unanimously accepted. There is debate about the timing of speciation events, with some studies indicating that pre-Pleistocene glacial cycles or geotectonic events were responsible for species diversification (Riddle 1996, Klicka and Zink 1997). Different species also responded differently to Pleistocene events and the challenge of post-glacial recolonisation. For example, dispersal patterns vary from species to species and this might have important effects on the scale of inter-population divergence and the relative importance of refugial versus leading edge expansion effects, resulting in different patterns of contemporary genetic subdivision (Ibrahim *et al.* 1996, Hewitt 2001, Tregenza *et al.* 2000, 2002). Other factors influencing species' responses to Pleistocene glaciations and subsequent post-glacial expansion include life histories and habitat requirements (Barrowclough *et al.* 2004, Geffen *et al.* 2004), the dominant mode of selection (natural vs. sexual) (Knowles 2000), whether the species in question were in arctic, temperate or tropical zones (Hewitt 2004) and how they responded to geographic barriers.

The Rocky Mountains have long been a source of interest to evolutionary biologists, given the potential for Pleistocene refugia in the area (Brunsfeld *et al.* 2001) and the presence of numerous hybrid zones formed upon secondary contact. Indeed, when Remington first introduced the idea of the suture zone – a particularly dense geographical clustering of hybrid zones – in 1968, he identified the Rocky Mountains as site of the second-largest purported suture zone in North America (Remington 1968). This idea went largely unchallenged for three decades, until Swenson and Howard (2004) performed an intensive reinvestigation of North American hybrid zones using GIS. They mapped hybrid zone hotspots using some of Remington's original data, and discovered low support for many of his original suture zones. However, they did find that two of their updated "hybrid zone hotspots" significantly overlapped with two of Remington's original suture zones. One of these was the Rocky Mountains.

The Rocky Mountains probably acted as a barrier to populations whose ranges were expanding out of eastern and western refugia during the Pleistocene (eg. Ayoub and Riechert 2004, Barrowclough *et al.* 2004). Inter- and intraspecific phylogeographic patterns often follow an east-west gradient in species distributed throughout the western and northern USA (eg. Orange *et al.* 1999, Riddle *et al.* 2000, Geffen *et al.* 2004, Hoffman and Blouin 2004), but the origins of current population genetic structure may be divisible into two components: older divergence due to separation into Pleistocene refugia (usually inferred from mitochondrial DNA sequences) and recent divergence due to drift and different selection pressures during post-glacial recolonisation (usually inferred from nuclear or genomic markers) (Bosart and Prowell 1998, Knowles and Richards 2005). The present study focuses on a katydid, the Mormon cricket (*Anabrus simplex*, Orthoptera, Tettigoniidae; Tettigoniinae), to gain a better understanding of how life history differences may have affected population genetic structure superimposed on deep refugial clades.

The range of Mormon crickets is bisected by the Rocky Mountains, and a recent mtDNA study uncovered two genetically distinct clades within the species that are approximately two million years old and mainly lie either side of the Rockies (Bailey *et al.* 2005a). These clades differ in many aspects of life history and behaviour, and broadly coincide with the two well-known phases of this flightless katydid which have been assumed to be phenotypically plastic (Cowan 1990, Gwynne 1984). Mormon crickets exhibit phases similar to migratory locusts, which have been termed "non-outbreak" and "outbreak" or "solitary" and "gregarious" (see Sword 2005 for discussion of semantics).

Solitary and gregarious populations inhabit the eastern and western slopes of the Rocky Mountains, respectively, and they have different mating systems (Gwynne 1981). Solitary individuals are small, cryptically coloured and sedentary, and males sing to advertise for mates. Gregarious individuals, however, are large, undergo destructive mass migrations and are darker in colour. Gregarious Mormon crickets are significantly more mobile than their solitary counterparts (Lorch and Gwynne 2000, Lorch *et al.* 2005). They exhibit coordinated, directional, long-range movement patterns and individual crickets have been shown to move up to 2 km per day, giving a potential monthly rate of 60 km (Cowan 1929, Lorch *et al.* 2005). In contrast, solitary populations are typically isolated in montane meadows of the eastern slope of

the Rockies, and their rates and distances of movement are significantly lower (Lorch *et al.* 2005). Dispersal is expected to have a homogenizing effect on genetic population structure by erasing any differentiation that may accumulate due to drift or selection (Lowe *et al.* 2004), though studies in birds demonstrated that non-random dispersal can in fact drive divergence (Coltman 2005). The North American migratory grasshopper *Melanoplus sanguinipes*, however, has been found to have very low levels of intraspecific genetic differentiation, probably as a result of long-range dispersal (Chapco *et al.* 1992, 1994). In Mormon crickets, the higher dispersal rate of the gregarious phase is expected to greatly increase the opportunity for gene flow in the western clade, which is comprised almost solely of gregarious populations (Bailey *et al.* 2005a), whereas the eastern clade is expected to show higher levels of population differentiation because it consists of spatially isolated, non-migratory solitary populations.

Solitary and gregarious Mormon crickets also exhibit other life history differences that may be predicted to affect population genetic structure. Gwynne (1984) demonstrated that sex role-reversal occurs in high density populations where food availability is restricted and the relative value of the male spermatophore is thus higher. Females compete aggressively for matings in such conditions, whereas males discriminate amongst potential mates, with the result that sexual selection pressure is likely to be stronger on females in gregarious populations but stronger on males in solitary populations (Gwynne 1984).

The present study investigates whether differences in life history between solitary and gregarious Mormon crickets predict differences in population genetic structure between the two clades. Given the likely Pleistocene origin of the division, we ask how such differences might influence patterns of genetic differentiation superimposed on the deep mtDNA division. Any mtDNA evidence of genetic structure resulting from recent events should be supported by similar nuclear patterns (Bossart and Prowell 1998, Knowles and Richards 2005) so in addition to producing an extensive mtDNA phylogeny across the study area, we also examine several nuclear microsatellite markers to test whether the phylogenetic patterns deduced from the mitochondrial genome correspond to those of the nuclear genome. First, we investigate the deep mtDNA division in Mormon crickets: is it a result of an ancestral population divided into east-west refugia, or is it better predicted by other factors, such as phase, that appear to correlate with the genetic division? Second, we investigate the causes of recent genetic structuring by testing the hypothesis that patterns of isolation by distance and population subdivision differ between

eastern and western clades as a result of dispersal differences. We also ask if there is a greater signal of population expansion in the gregarious clade, which would be expected to have a history of population outbreaks.

Materials and Methods

Sampling

A total of 16 gregarious populations, 10 solitary populations and 1 that was intermediate in phase were sampled between 1999 and 2004. Mormon crickets intermediate in phase have characteristics of both gregarious and solitary populations: they occur at high densities but are sedentary and do not migrate (Gwynne, pers. obs.). Several of the populations in this study were sampled in more than one year (some Tettigoniid species lay eggs with variable diapause lengths and hatching can occur over several seasons: Hockham *et al.* 2001). Figure 1 shows the locations of the sampled populations. Gregarious populations were sampled from northwestern Colorado and central Utah. Solitary populations were mainly sampled from the eastern slope of the Rockies in central Colorado, although there is one notable exception: the Little Brush Creek population is from northwestern Colorado but is solitary. The intermediate-phase Mormon cricket samples from Montana—the most geographically isolated population in this study—are roughly 1,000 km from the other populations (see figure 1 and Gwynne 1993).

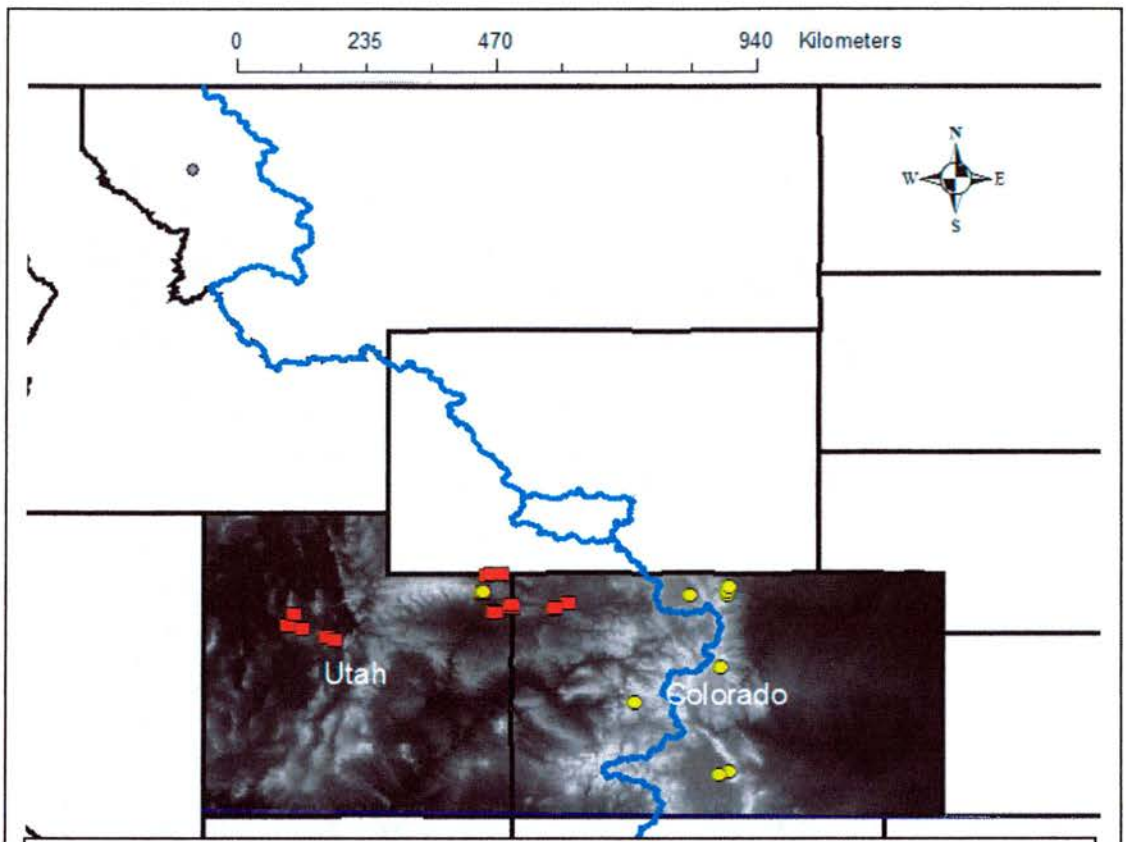


Figure 1: Map of populations sampled. Solitary populations are represented by yellow circles and gregarious populations are represented by red squares. A grey circle denotes the Montana population, and the continental divide is in blue.

MtDNA analysis

DNA was extracted using the PureGene protocol (Gentra Systems, Minneapolis, Minn.), and segments (totaling 850 bp) of two mitochondrial genes, COII and COIII, were amplified using the primers and PCR profile detailed in Bailey *et al.* (2005b). We sequenced the amplified products using a Beckman-Coulter automated sequencer. It was prohibitively expensive to produce both forward and reverse sequences for each individual and gene, so all samples were forward-sequenced and a subset (n=10) for both COII and COIII were reverse-sequenced to ensure that forwards and reverses matched. They all did.

Eighty-three individuals were successfully sequenced for both loci, and we edited and aligned these sequences using Chromas 2.12 (Technelysium, 2001) and ClustalX 1.81 (Thompson *et al*, 1997). A partition homogeneity test in PAUP* (Swofford 1998) was used to

establish that the phylogenetic signal from each gene was congruent, thus COII and COIII fragments were concatenated and analysed together. These eighty-three new sequences were combined with 32 from a previous study (Bailey *et al.* 2005b) to produce a final sample size of 115 samples from 26 populations. Five populations overlapped the two studies.

We used the program Network v.4.1.1.1 (Bandelt *et al.* 1999) to construct a median-joining haplotype network for our Mormon cricket samples. Differences in metapopulation structure may be more easily visualized in networks than in traditional phylogenetic trees, with star-shaped haplotype clusters indicating recent rapid population or range expansions and long, branched networks indicating greater genetic structure (Avise 2000).

A matrix of population pairwise genetic distances (Slatkin linearised F_{st}) was produced in Arlequin ver. 2.000 (Schneider *et al.* 2000). These genetic distances were then used in conjunction with population pairwise geographic distances calculated using a surface distance calculator (http://www.wcrl.ars.usda.gov/cec/java/lat-long.htm) to test for isolation by distance using the Isolde option in GenePop v.3.4 (Raymond and Rousset 1995). Isolation by distance (IBD) was tested separately among populations comprising the eastern clade and among populations comprising the western clade. We tested for differences in patterns of IBD in the mtDNA data by testing whether the slope of the regression of F_{st} on geographic distance differed between the eastern and western clades using a general linear model (p values from this should be taken as approximate, because this does not account for the interdependence of the distance data, but there is no Mantel test equivalent of an interaction term).

We used a partial Mantel test to assess how well several variables predict mtDNA genetic distance in our samples, including phase and geographic distribution. The test is similar to a multiple regression, but evaluates the relative importance of one variable while at the same time controlling for the remaining ones. The full set of independent variables considered were geographic distance, phase (solitary, gregarious, or intermediate as 0, 1 and 0.5, respectively), whether the populations fall east or west of the continental divide (0 or 1), elevation, and average yearly precipitation (from National Resources Conservation Service, USDA http://www.ncgc.nrcs.usda.gov/products/datasets/climate/index.html). The partial mantel test was run in FSTAT v.2.9.3 (Goudet 2001).

The 'outbreak' nature of the gregarious form could be expected to lead to less population stability, with more periodic extinction and expansion. Mismatch distributions between the

haplotypes are expected to have a characteristic 'ragged' shape in stable populations, but a smooth peak (at a location related to the time since the most recent expansion) in an expanding population (Harpending 1994, Excoffier 2004). Mismatch distributions were calculated separately for the two clades in DnaSP (4.10) (Rozas *et al.* 2003). Values for an expected distribution were generated by setting the final population size to be effectively infinite, allowing calculation of an observed theta (initial population size) and tau (time since expansion times mutation rate).

Microsatellite analysis

Microsatellite loci in *A. simplex*, as in many Orthopteran insects, occur at low frequencies and are susceptible to null allele problems (see Bailey *et al.* 2005b). Here we constrain our analysis to four out of the seven highly polymorphic loci developed in *A. simplex* that cleanly amplify and have the fewest null alleles, based on deviations from HWE (Bailey *et al.* 2005b). These were loci 146, 383, 782 and 1710. We screened 593 individuals from 17 populations, of which three were sampled twice—once in 2001 and again in 2004. Sample sizes ranged from 8 to 62 individuals, and the PCR conditions used were as described in Bailey *et al.* (2005b). Most were scored using a Beckman-Coulter automated sequencer, although some were scored on PAGE gels. We calibrated PAGE gel scores by running several of the same samples on both. The number of loci scored is necessarily modest but provide an important test of whether the patterns seen in the mtDNA are replicated in the nuclear genome.

We first assessed whether populations were in Hardy-Weinberg equilibrium at each locus and across all four loci using Genepop v.3.4 (Raymond and Rousset 1995). One locus—146—was discarded from subsequent analyses because of deviation from equilibrium in 75% of the populations studied; however, patterns of isolation by distance were consistent between this locus and the other three, which were in HWE. Using the remaining three loci, we created a population pairwise matrix of Slatkin's linearised R_{st} values with Genepop v.3.4 (Raymond and Rousset 1995). Isolation by distance was assessed for the microsatellite data using the Isolde option in Genepop v.3.4 (Raymond and Rousset 1995). As with the mtDNA analyses, we separately tested isolation by distance among populations within the eastern and western mtDNA clades. We tested whether the slope of the regression of R_{st} on geographic distance differed

between the eastern and western clades using a general linear model (again, p-values are approximate). Finally, we recalculated population pairwise R_{st} comparisons by sex in the western clade, and tested whether there was a significant difference in average pairwise R_{st} between males and females. Smaller sample sizes precluded us from doing this analysis in the eastern clade.

Results

mtDNA phylogeny

Figure 2 shows the network produced from concatenated COII and COIII mtDNA sequences. There is a broad genetic division that corresponds to an "eastern" clade comprised of solitary populations from the eastern slope of the Rockies, and a "western" clade that is predominantly gregarious (also seen in a previous study, Bailey *et al.* 2005a). The solitary individuals that fall into the "western" clade are all from Little Brush Creek, UT. This population is geographically located with the gregarious populations in the western portion of our sampling area, in a mountain meadow with rich habitat structure (see figure 1). Also noteworthy is that the intermediate-phase Polson, MT samples are scattered throughout the "western" clade, even though they are geographically separated from all other populations by approximately 1,000 km. The most striking feature of the network is the greatly increased distances between the eastern haplotypes, contrasting with the dense, star-shaped cluster of haplotypes from the west.

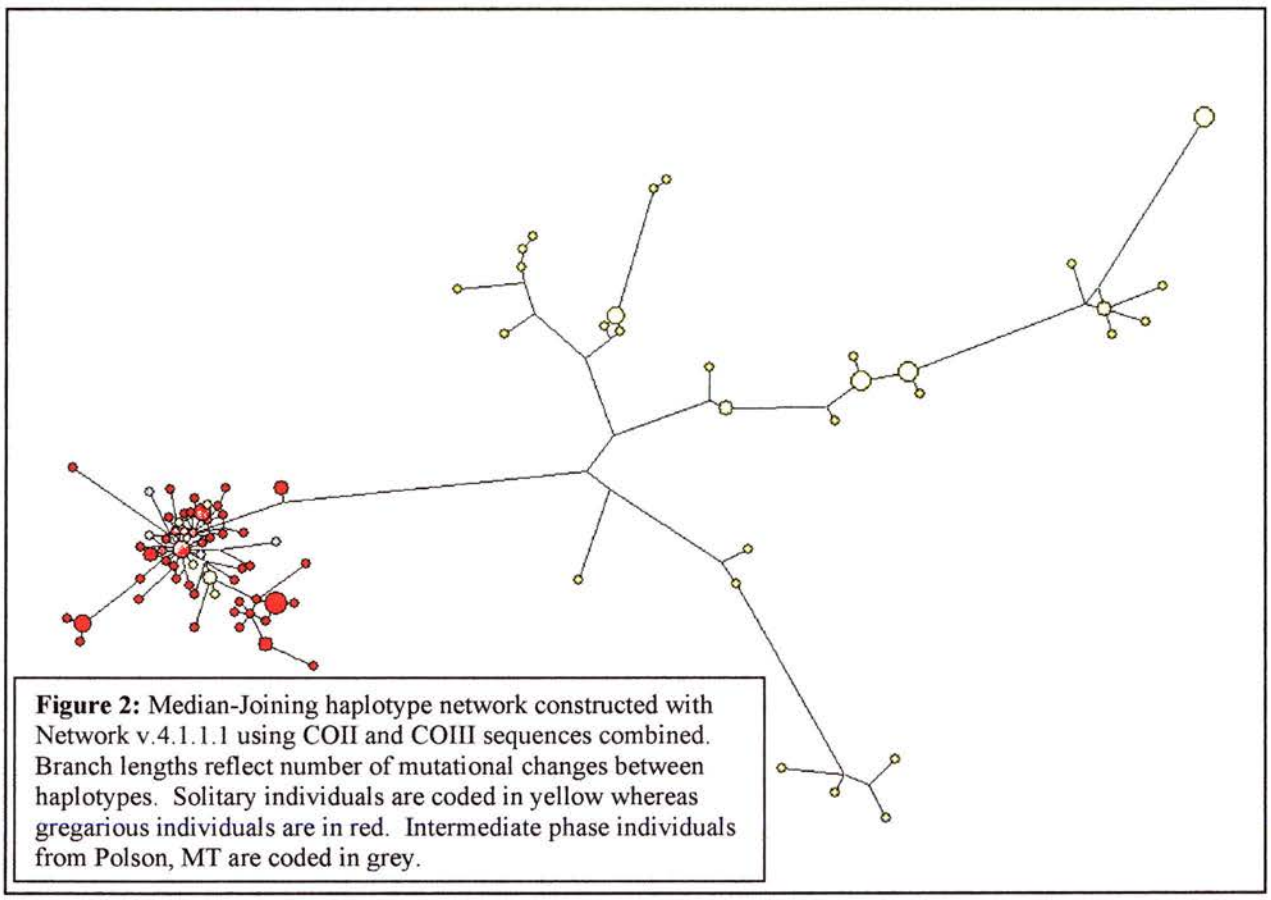


Figure 2: Median-Joining haplotype network constructed with Network v.4.1.1.1 using COII and COIII sequences combined. Branch lengths reflect number of mutational changes between haplotypes. Solitary individuals are coded in yellow whereas gregarious individuals are in red. Intermediate phase individuals from Polson, MT are coded in grey.

Microsatellite data

Only one population at one locus (locus 782) was out of HWE after a Bonferroni correction (p significant at 0.002). Similarly, across all loci, only two populations were out of HWE after a Bonferroni correction (p significant at 0.002). Bonferroni and sequential Bonferroni corrections may increase the probability of type II error, however, which in the present situation makes the test less conservative (Moran 2003). We calculated HWE without a Bonferroni correction and for each population at each locus, 7 of 63 populations (of which 4 were gregarious) were out of HWE. Across all loci, 6 of 21 populations (of which 5 were gregarious) were out of HWE in the absence of a Bonferroni correction.

Isolation by distance

Isolation by distance was calculated separately for each marker, across all the samples and separately within each clade. Table 1 shows the results of these tests. Both sets of markers indicated that isolation by distance was significant when all populations were being compared. However, when the analyses were separated by clade, significant isolation by distance was only observed within the eastern clade for mtDNA, although it was nearly significant in the western clade. Isolation by distance for the microsatellite data was observed in neither the eastern nor the western clade (Table 1).

The slopes of the regression of genetic distance (Slatkin linearised F_{st} or linearised R_{st}, for mtDNA and microsats respectively) on geographic distance were significantly different between the clades (figure 3) as indicated by significant interaction terms between distance and clade in general linear models ($F_{1,168}=23.98$, $p<0.001$, $F_{1,122}=23.50$, $p<0.001$, for mtDNA and microsatellites respectively).

Table 1: Isolation by distance. Significant p-values are in bold, indicating that the populations examined were isolated by distance.

	Overall	Eastern clade populations only	Western clade populations only
mtDNA	**P< 0.001**	**0.006**	0.055
microsatellites	**0.008**	0.163	0.621

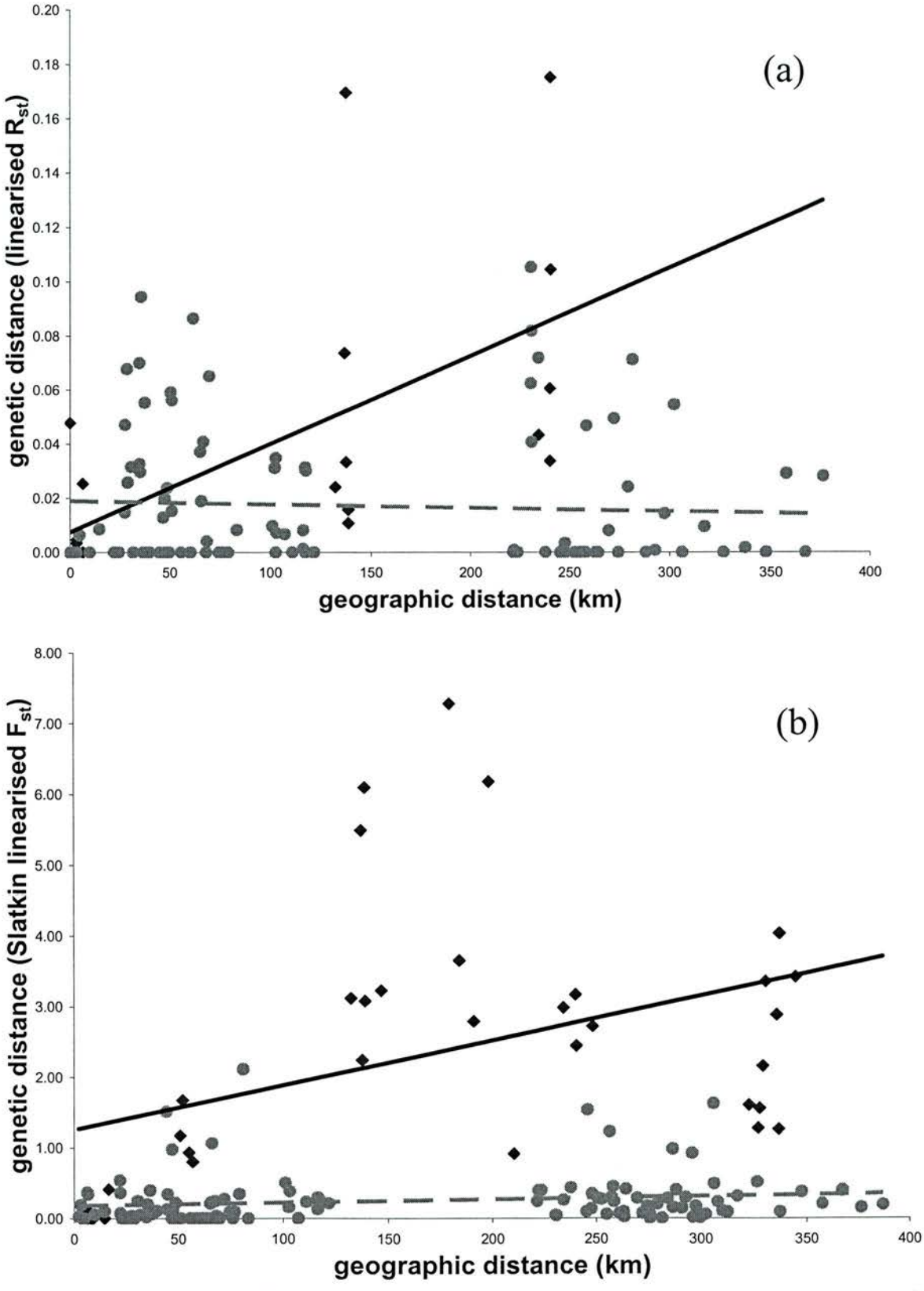


Figure 3: The slope of the regression of genetic distance (linearised R_{st} or Slatkin linearised F_{st}) on geographic distance is significantly different between eastern clade comparisons (black line) and western clade comparisons (dashed line) for both **(a)** the microsatellite analysis and **(b)** the mtDNA analysis (GLM: $F_{1,168}=23.98$, $p<0.001$ and $F_{1,122}=23.50$, $p<0.001$, respectively).

Partial Mantel Test

The partial mantel test indicated that only two of our five predictor variables were significantly correlated with mtDNA variation (Table 2). These were geographic distance and whether the populations fell east or west of the continental divide. Elevation, precipitation, and phase were not significant (Table 2).

Table 2: Partial Mantel Test results, giving Beta and P(Beta) for each predictor variable. Significant variables are in bold.

Variable	*Beta*	*P(Beta)*
Geographic distance (km)	**0.18**	**0.017**
East or west of divide	**1.00**	**<0.01**
Precipitation	0.10	0.087
Phase	0.21	0.22
Elevation (m)	-0.07	0.36

Mismatch distribution

Figure 4 shows the observed mismatch distribution plots for each clade. The haplotypes from the east show the characteristic "jagged" distribution expected of an old stable population whereas those from the west have a single broad peak, and have a lower average level of mismatch. These results are compatible with an older and stable population structure in the east but a more recent expansion in the west. Figure 4 also shows the expected distribution generated for the values of theta (initial population size) and tau (time since expansion times mutation rate) given in Table 3. The distribution seen in the western clade is compatible with a model of recent population expansion, but not the eastern clade. Table 3 also gives the results of 1000 replicate coalescent simulations of Fu's F test for neutrality or population expansion (Fu 1997) which rejects the stable population size model for the western, but not the eastern clade. These data suggest that the western clade is derived from a population which expanded within the last few thousand to 200,000 generations ago, depending on mutation rates, which is compatible with a recent to Pleistocene time scale.

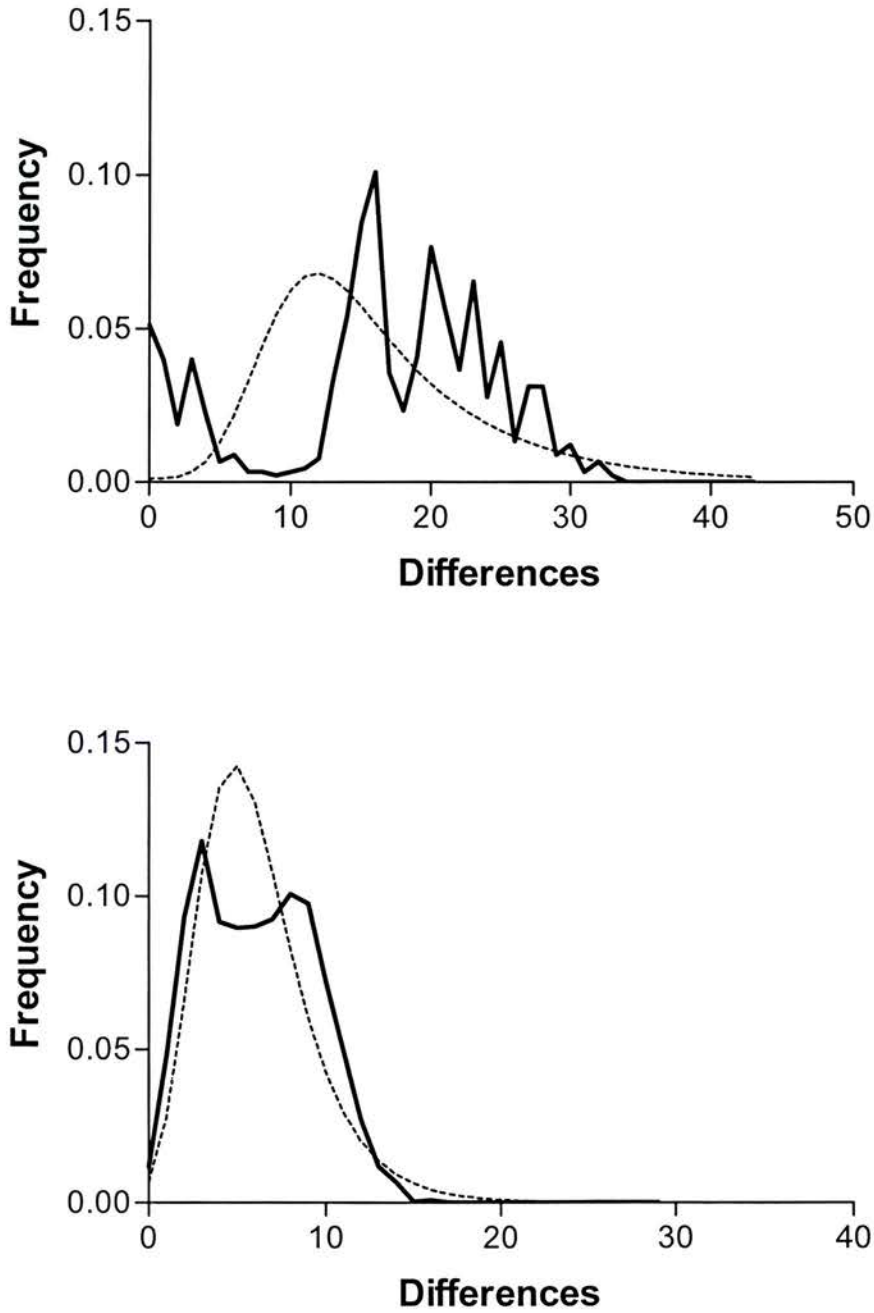


Figure 4: Mismatch distribution plots for the two clades, east (top) and west (bottom). The solid line is the observed distribution, the dotted that expected allowing for an expanding population size.

Table 3: Values of initial theta and tau used to derive the expected distributions given in Fig 4. These were generated by assuming a large current effective population size (see Rozas *et al.* 2003). 95 % confidence intervals and p values from Fu's F test were generated by coalescent modelling.

	Initial theta	tau	Fu's F (CI, p)
East	7.215	9.150	-0.03 (-6.36 - 6.64, ns)
West	2.083	4.036	-56.56 (-5.12 – 5.70, <0.0001)

Sex differences in RST

The mean linearised R_{st} for gregarious females was 0.0098, but 0.0030 for males, a three-fold difference. This was significant (Mann-Whitney U Test: W=9612.0, $p<0.001$; the data were non-normal).

Discussion

This study confirms the existence of two deep (ca. 2 million years old) mtDNA clades within Mormon crickets (Bailey *et al.* 2005a) that broadly coincide with the two well characterised phases of the animal. Knowles and Richards (2005) uncovered genome-wide genetic structuring in the Rocky Mountain-distributed grasshopper *Melanoplus oregonensis* that indicates older divisions corresponding to refugial population structure, plus more recent drift-induced differences that may have been subject to further divergence through selection. Here we examine the causes of recent genetic structure superimposed on a deep phylogeographic division by assessing the geographic and life history correlates of the eastern and western clades using mtDNA sequences contrasted with a modest sample of highly polymorphic nuclear markers.

This study provides strong evidence that the Rocky Mountains acted as a wedge, separating *A. simplex* into eastern and western refugia during Pleistocene glaciations. Aside from geographic distance, the only other significant predictor of genetic distance in a partial Mantel test was whether the sample was east or west of the continental divide. The east-west division had a clear and marked predictive value, even exceeding geographic distance in significance, and we were able to reject other factors such as precipitation, elevation and phase as contributing to the genetic division. The latter point is of interest, because the broad correspondence between phase and the two genetic clades could be misconstrued as a causal

relationship when in fact our results indicate that phase has very little predictive value for genetic distance.

This finding and the timing of the division is supported by the known paleoclimatic history of this region. The southernmost extent of the Laurentide ice sheet did not advance into the Colorado Rockies during the Wisconsin glaciation; however, portions of the Rockies and the Pacific northwest experienced ice cover and various degrees of continuous and discontinuous permafrost (Williams *et al.* 1998). Even if speciation was initiated during the Pliocene, glacial events during the Quaternary are likely to have reinforced lineage separations, as has been found in some avian species (Avise and Walker 1998). Repeated Pleistocene glacial cycles are thought to have promoted divergence as species were displaced from the Rockies and other areas in the United States into refugia (Hewitt 2000, Brunsfield *et al.* 2001, Knowles 2001) or their habitats were fragmented by ice sheets (Small *et al.* 2003, Weir and Schluter 2004). Such temperate refugia typically result in deep genetic divisions, indicating that genetic differences have accumulated over several ice ages (Hewitt 2004). Rocky Mountain orogeny occurred approximately 4 million years ago, and in line with other studies that have found Pleistocene glaciations to be a promoter of divergence (eg. Knowles 2001, Brunsfeld *et al* 2001), the timescale over which Mormon crickets have diversified into discrete eastern and western clades suggests that the Rockies acted as a barrier that a) forced retreating populations into separate refugia and b) may have prevented secondary contact and gene flow during interglacials.

Such east-west patterns of genetic divergence in this region and across the continental US are well-documented and often attributed to Pleistocene vicariant events: for example in the squirrel *Sciurus aberti* (Lamb *et al.* 1997), the mouse *Peromyscus eremicus* (Riddle *et al.* 2000), the Northern Leopard frog *Rana pipiens* (Hoffman and Blouin 2004), the grey wolf *Canis lupus* (Geffen *et al* 2004) and the desert lizard *Gambelia wislizenii* (Orange *et al.* 1999). The desert spider *Agelenopsis aperta* also shows genetic structure in an east-west transect of putative refugia; however, Ayoub and Riechert (2004) make a distinction between the ultimate causes of such genetic divergence, positing that divergence between eastern and western populations resulted from variable responses to conditions during repeated glaciations, as opposed to restricted gene flow across the Rockies. Results from Mormon crickets support the conclusions of Knowles and Richards (2005): these two scenarios are not necessarily mutually exclusive.

It is possible that overall patterns of intraspecific population differentiation can obfuscate local-scale or population-level patterns that could be useful to elucidate the evolutionary history of a species (Avise 2000, p152) so we subdivided our analyses by eastern and western clade. Given that the two clades were most likely isolated in separate refugia during the Pleistocene, and thus experienced differing conditions during repeated glaciations and post-glacial expansion, subsequent analyses were focused on comparing more recent patterns of genetic differentiation between the eastern and western clades.

Despite the fact that the clades reflect refugial divergence, it remains true that most samples from the west are gregarious and all those from the east are solitary. If this has been a consistent situation for thousands of generations, the large differences in life history, notably migratory behaviour and population explosions during outbreaks, might be expected to have influenced population genetic structure. There is clear evidence that this is the case. The fact that "intermediate" phase *A. simplex* samples from Polson, MT (separated from the other populations by roughly 1,000 km) fall out within the western clade is an important and unexpected result because it provides evidence that demographic or life history differences have had a stronger effect than geographic distance on the opportunity for gene flow within the western clade.

Geographic distance is a strong predictor of genetic distance in the all-solitary eastern clade, whereas geography seems to have very little influence on population structure in the western clade (figure 3). Bailey *et al* (2005) also found by AMOVA that more mtDNA differentiation was partitioned amongst populations in the eastern clade. Differences in dispersal distance and rate between solitary and gregarious Mormon crickets are significant (Lorch *et al.* 2005), and the star-shaped polytomies in our western clade support the hypothesis that migration in gregarious, but not solitary, Mormon crickets has had a homogenizing effect on the population genetic structure in the western clade. The opportunity for migrating bands to come into contact is high; there have been accounts of separate bands meeting and either crossing through each other to continue on in their respective directions, or coalescing and moving onwards as a single, larger band (Cowan 1929, Sorenson and Jeppson 1940, MacVean 1987).

The mismatch distribution for the western clade conformed to a model of recent population expansion, which further supports the position that demographic differences have influenced recent metapopulation genetic structure. Gregarious Mormon crickets' tendency for almost explosive outbreaks and rapid, long-range dispersal is correlated with a population

structure in the western clade dominated by fewer haplotypes which are more widespread geographically. A similar situation is observed in the migratory grasshopper *Melanoplus sanguinipes*, in which recent colonisations and a high reproductive capacity may have driven cycles of population explosions and crashes, resulting in high gene flow that has prevented genetic differentiation over a wide geographic range (Chapco *et al.* 1992, 1994). Differences in dispersal ability may also be related to differences in tolerance for certain habitats. Gregarious bands have been observed in previously forested areas that have been recently burned, and crossing juniper-forested hills (Bailey, pers. obs., Sorenson and Jeppson 1940). In contrast, solitary populations are confined to montane meadow habitats and have little opportunity for dispersal. Field observations suggest that populations in the western clade seem more able to cope with different habitats because of their tendency to move through them quickly, and combined with dispersal ability this may have influenced the opportunity for historical population expansions.

Both sets of data produced similar isolation by distance results in this study, so our conclusions are not based solely on a uniparentally inherited marker that may indicate different patterns for males and females. Sex-biased dispersal in gregarious Mormon crickets has not been observed in radio-tracking experiments (Gwynne and Lorch, pers. comm.), but in the western clade, between-population F_{st} in females was three times as high as that in males. It is possible that females simply have less time to migrate after mating because they must consume a large nuptial gift (the spermatophore) and oviposit, whereas males may be motivated to move onwards in search of food, in part to regenerate glandular material to produce additional gifts and seek other females. However, it remains curious that any such differences were not detected by radio tracking yet are clearly detectable at the genetic level.

The results of the present study show evidence of historical demographic differences between populations in different refugia, which could be a result of differential responses to Holocene conditions following vicariance during Pleistocene glaciations. In Mormon crickets, lower genetic differentiation and the footprint of population expansion in the western clade but not the eastern one echoes patterns observed in other species. A study of the American marten (*Martes americana*) revealed greater genetic differentiation in coastal refugial populations resulting from habitat fragmentation and isolation during the Holocene, whereas a second more widespread refugial clade experienced recent expansion-contraction cycles which increased gene

flow and reduced population genetic differentiation (Small *et al.* 2003). Further evidence that climatic changes affected eastern and western refugial populations variably comes from the sharp-shinned hawk *Accipiter striatus velox* (Hull and Girman 2005). Mismatch distributions in this species indicate that populations in the west experienced an expansion relative to those in the east during the Holocene hypsithermal dry period (Hull and Girman 2005). Northern Leopard frogs also show evidence of recent range expansions and reduced genetic diversity in populations west of the Mississippi river, which is likely to have acted as a barrier to gene flow in this species (Hoffman and Blouin 2004). Finally, a recent expansion-contraction cycle in the painted turtle *Chrysemys picta* has been suggested in the Rocky Mountain and Great Plains region and was attributed to the retreat of the Laurentide ice sheet approximately 14,000 years ago (Starkey *et al.* 2003). Patterns of genetic differentiation within refugial phylogroups are likely to depend on the species-level interaction between Holocene climatic events and geographic features of the refugia, but our results concur with findings in these species, and suggest that intrinsic geographic properties of western refugia may have provided a setting that was more conducive to population expansion-contraction cycles during the Holocene. Factors that promote population instability and repeated expansion-contraction cycles, for example environmental fluctuations interacting with geographic features, may have been more severe in western refugial ranges and are expected to select for increased flexibility in the response to environmental fluctuations. The paleoclimatic history experienced by the western clade in Mormon crickets may have thereby selected for long-range dispersal ability, i.e. gregariousness, or a decrease in the plastic threshold response to environmental conditions that induce gregariousness.

Acknowledgements

Funding for fieldwork was generously supported by grants from the Genetics Society, the Linnean Society Systematics Research Fund, the British Ecological Society and the St. Andrews Russell Trust. Molecular labwork was funded by MGR and the University of St. Andrews and support for NWB was provided by an Overseas Research Scholarship and the University of St. Andrews. The authors owe a debt of gratitude to William Bailey for good-humored and valuable assistance in the field.

Do Solitary and Gregarious Mormon Crickets Differ in Calling Song?

NW Bailey, WV Bailey, DT Gwynne, MG Ritchie

Abstract

Male courtship song traits are likely to diverge quickly in allopatric populations due to drift, and the evolution of differences in parameters such as carrier frequency, chirp rate, and reluctance to sing can even precede sexual isolation. Mormon crickets exist in two phases, solitary and gregarious, the latter of which are typically sex role reversed, and these phases are largely correlated with a significant allopatric mtDNA subdivision with the species. Both allopatric divergence and sex role reversal are hypothesized to have had an effect on male courtship song in solitary and gregarious populations, and we examine several male song parameters to test the prediction that gregarious males show distinguishable differences in both morphological and song traits, and that they are more reluctant to sing than solitary males. We also tested for a difference in the relationship between mirror size and carrier frequency, as some models of sound production indicate that mirror size should predict carrier frequency. Results supported our hypotheses. A multivariate canonical discriminate analysis showed that solitary and gregarious individuals were completely distinguishable across multiple song and morphological measurements. Carrier frequency differed between the two phases, and gregarious males were more reluctant to sing overall. Mirror size predicted carrier frequency; however, the relationship between mirror size and surface area varied between solitary and gregarious crickets, suggesting that factors above and beyond mirror size predict carrier frequency. Coupled with the genetic divergence between the populations studied, these results suggest that solitary and gregarious Mormon crickets differ in a broad suite of traits that may suggest subspecies status.

Introduction

Sexually selected traits—especially male courtship song—have been suggested to be particularly susceptible to divergence in allopatrically isolated populations (West-Eberhard 1983), and sexual selection on courtship song has been proposed to be a significant driver of speciation (Andersson 1994, Henry 1994, Mendelson and Shaw 2005). Differences in male courtship song may evolve particularly rapidly, sometimes even more rapidly than sexual isolation (West-Eberhard 1983, Gleason and Ritchie 1998, Tregenza *et al.* 2000), and secondary sexual traits such as male song may also be particularly responsive to drift (Coyne and Orr 1989, Roff *et al.* 1999). Divergence in such characters may thus be an indicator of subspecies or incipient speciation events (Butlin and Ritchie 1994, Ritchie *et al.* 1997, Gleason and Ritchie 1998, Roff *et al.* 1999).

The most common mechanism for singing in insects is stridulation, where specialised areas of the body are made to reverberate by striking against stridulatory pegs (Ewing 1989, Greenfield 1990). In katydids, the tegmina are modified so that during each wing-stroke, pegs on the stridulatory file of the upper (left) elytra strike the plectrum on the lower (right) elytra, causing the mirror, a circular membrane situated next to the plectrum, to vibrate (Ewing 1989, Greenfield 1990). In some Ensiferan species, resonance of the mirror (or harp in gryllids) is not damped between peg strikes and calls are sustained, or "trilled". However, most katydids do not trill because resonance from each peg strike is highly damped before the onset of the next peg strike.

Several models have been developed to predict the factors most likely to control the dominant or carrier frequency of song, but most of this work has been done in trilling gryllids. Under the "clockwork cricket" model of stridulation developed from studies of *Gryllus campestris* (Elliott and Koch 1985, Koch *et al.* 1988), the dominant or carrier frequency of the song is determined by the resonant properties of the harp (the gryllid equivalent of the mirror), and the stridulatory file represents an escapement mechanism whereby the force (peg strike) causing resonance of the oscillator (harp) is delivered at evenly spaced time intervals due to the incremental slippage and striking of pegs upon the plectrum in much the same way a clock escapement functions. In trilling crickets, this escapement mechanism ensures that peg strikes

are optimally phased to the fundamental resonant frequency determined by the mirror (Bennet-Clark and Bailey 2002). This model has been criticised however, and an alternative model proposes that the subalar air space also acts as a resonator, and that phasing of peg strikes is under neural control (Stephen and Hartley 1995, though see Prestwich *et al.* 2000).

Mormon crickets (*Anabrus simplex*, Orthoptera, Tettigoniidae) are flightless katydids ranging throughout the American west that occur in solitary and gregarious populations. Solitary individuals mainly inhabit the eastern slope of the Rocky Mountains and are cryptically coloured, whereas gregarious populations are more widely distributed and form destructive migratory bands that can travel up to 2 km per day (Cowan 1990, Lorch *et al.* 2005). Their dark aposematic colouration and banding behaviour reduces the risk of predation (Sword *et al.* 2005) and they may occur at densities over a thousand times higher than in solitary populations (Gwynne 1984).

Solitary and gregarious Mormon crickets also have different mating systems. Gwynne (1981) demonstrated that Mormon crickets in high density, food limited conditions show role reversal, with females competing for matings with discriminating males. Male Tettigoniids transfer a spermatophore to females upon mating (Gwynne 2001), and this "nuptial gift" represents a relatively greater paternal investment when the food availability is low—as is the case in the dry sagebrush habitats where gregarious outbreaks occur—which biases the OSR towards females and reverses courtship roles (Gwynne 1981, 1984, 1993). Song traits were also found to vary in *Ephippiger ephippiger* under experimental conditions of sex role reversal (Ritchie *et al.* 1998). Role reversed *E. ephippiger* males sang less frequently and at a lower intensity, but female preferences remained static (Ritchie *et al.* 1998). Studies examining differences in male song in naturally-occurring role-reversed populations are less conclusive. In another Tettigoniid, *Metaballus litus*, role reversed males exhibit a different type of song (Gwynne 1985), and in *Kawanaphila nartee*, fewer males were observed to sing in populations that did not have access to pollen (Gwynne *et al.* 1998) which is correlated with an increase in the size of male spermatophores (Simmons and Bailey 1990). OSR, and thus the proportion of singing males, in *K. nartee* varies over short geographical distances due to microhabitat differences between populations (Gwynne *et al.* 1998). In natural populations of Mormon crickets, males at low-density (i.e. solitary) sites called for longer periods of time than those at

high-density (i.e. gregarious) sites (Gwynne 1984) but neither reluctance to sing (i.e. the incidence of singing males) nor structural differences in song have been investigated.

It has been assumed that the differences between solitary and gregarious forms reflect a phenotypically plastic response to environmental conditions (Gwynne 1984), however, more recent mtDNA studies have uncovered a significant genetic division within the species that broadly corresponds to western gregarious populations and eastern solitary populations (Bailey *et al.* 2005). Molecular clock arguments suggest that this division is approximately 2 million years old, and the two lineages likely represent discrete evolutionary histories (Bailey *et al.* 2005). Divergence in courtship song is predicted given that such a degree of genetic divergence is consistent with subspecies (Hewitt 1996, Lunt *et al* 1998), and song divergence may evolve quickly in allopatrically isolated populations.

Sex role reversal and allopatric divergence are hypothesized to have had an effect on male courtship song in solitary and gregarious populations, and we examine several male song parameters to test the prediction that gregarious males show distinguishable differences in both morphological and song traits, and that they are more reluctant to sing. In line with Gwynne's (1984) observation that sexual size dimorphism is more pronounced in gregarious populations (with females larger than males), relaxed sexual selection on gregarious males should increase their variance in body size relative to that of gregarious females and solitary males, both of which we predict to be constrained by greater sexual selection. We also examine the relationship between mirror surface area and carrier frequency to see whether it is constant between solitary and gregarious phases.

Materials and Methods

Study populations

Three gregarious populations were sampled in north-western Colorado near Dinosaur National Monument and are coded LE, CO and TM. Solitary populations were sampled at Kelly Flats (KF) and Indian Meadows (IM) in the Poudre Canyon on the eastern slope of the Colorado Rockies. Due to their highly discontinuous distribution, the three gregarious populations were about 300 km away from the two solitary populations.

A range of morphological and behavioural traits (described below) were measured on all individuals. We used a multivariate discrimination analysis (with cross-validation) to assess whether animals from the two clades were easily distinguishable, and we used an unbalanced nested general linear model (GLM) on the first canonical variate scores to test whether we could combine populations within phase for subsequent analyses.

Reluctance to sing

'Reluctance to sing' experiments were performed in two separate summers: June and July of 2004 and 2005. In both years, adult solitary males were captured from KF and IM, and adult gregarious males were captured from LE. Subjects were fed an excess of lettuce, yellow sweetclover (*Melilotus officinalis*) and seed heads from grasses, and kept in same-sex cages with approximately 15 individuals for at least three days, to ensure that males who may have mated previously replenished their protein reserves.

In 2004, reluctance to sing was assessed by placing individual males in a cylindrical mesh cage (approx. 30 cm in diameter and 45 cm long) placed half in sunlight and half in shade

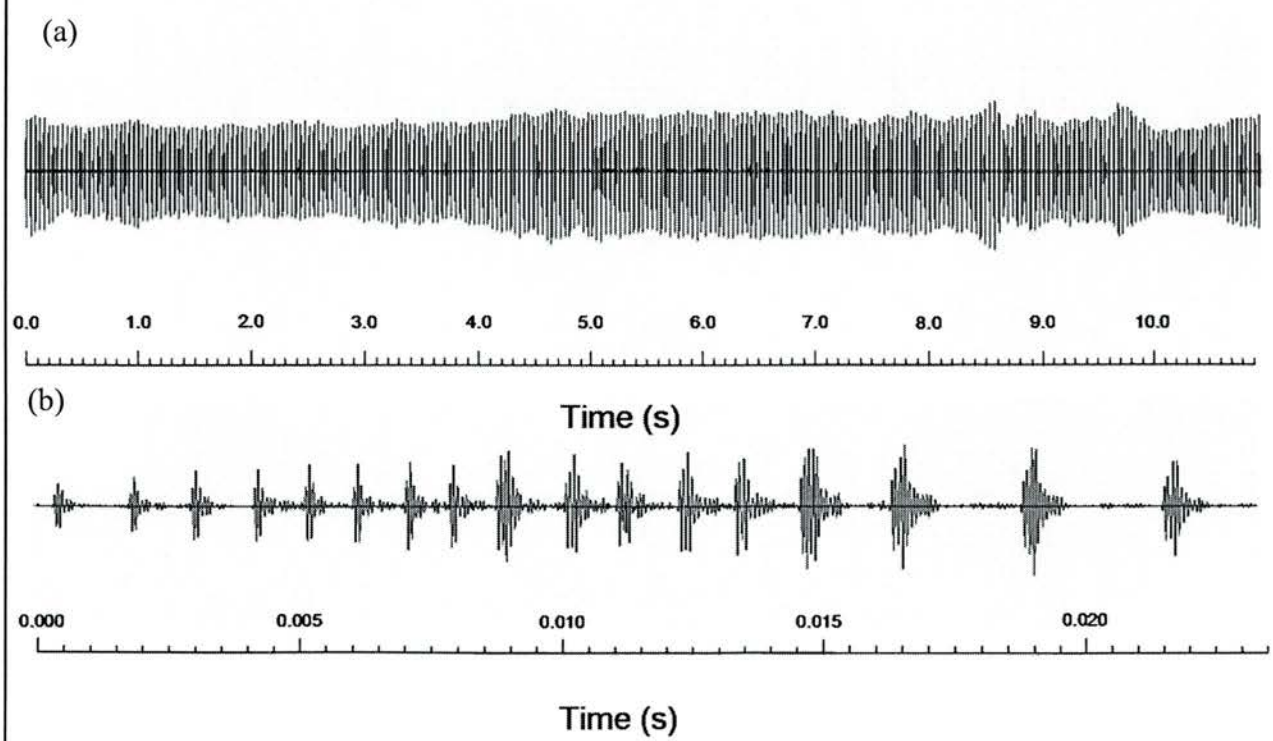


Figure 1: Oscillograms of Mormon cricket song showing **(a)** sustained singing and **(b)** damping of mirror oscillations between peg strikes.

in an enclosed chamber. Mormon crickets can sing continuously for many consecutive minutes, or song can be broken into shorter bouts of singing. Individual chirps are produced during the closing motion of the wings and resonance produced by mirror vibrations at each peg strike is highly damped (Figure 1). Calling activity was recorded for 10 minutes using a Sennheiser ME 66 directional microphone with a K6 powering module. Recordings were made digitally using Sony Sound Forge 7.0a software (Sony Pictures Digital Inc. 2003) installed on a laptop computer in the field, and recording began as soon as the experimenter moved out of sight. Temperature was noted. We used 24 solitary males and 24 gregarious males, chosen randomly, and all trials were run between 8:30 am and 1:00 pm when Mormon crickets are most active. In 2005, the experiment was performed in the field as close to the original population as possible but far enough away to be out of hearing range of other singing crickets (approx. 1 km). The mesh cage (approximately 10 cm in diameter and 25 cm long) was placed in a clump of sagebrush, half-shaded and half in the sun, and the rest of the experiment was as before. Seventeen gregarious males and 10 solitary males were used. We tested whether the incidence of singing versus non-singing males differed between solitary and gregarious populations and between years using a 3-factor G test (Sokal and Rohlf 1969).

In-situ recordings

In a separate experiment, gregarious males were recorded in the field from LE, TM and CO (n=16, n=8 and n=16) and solitary males were recorded from IM and KF (n=10 and n=11) during June and July of 2005 with a Sennheiser ME 66 microphone as before. Care was taken not to disturb the crickets while they were being recorded. The temperature at the time of recording was noted, and after each male stopped singing it was captured and preserved in ethanol. Songs were analysed using Sony Sound Forge 7.0a (Sony Pictures Digital Inc. 2003). We used a sample rate of 96 kHz and the microphone had a nominal frequency response of 50 Hz to 20 kHz. Average carrier frequency (C_f) was determined by analysing five one-quarter second samples per individual using a Fast-Fourier Transform (FFT) of size 32,768 with a Blackman-Harris smoothing window.

To determine what factors predicted C_f, we used a full GLM with temperature, head capsule width (HCW), phase and mirror surface area as the independent variables. Mormon

cricket mirrors are approximately circular (figure 2), and the frequency produced by a circular membrane is expected to vary according to the following equation:

$$f = [k/D]*[\sqrt{(T/\sigma)}] \qquad (1)$$

where k is a constant, D is diameter of the membrane, T is tension of the membrane and σ is the density of the membrane (Sears and Zemansky 1964). If T and σ remain constant, D can be expressed in terms of surface area (S_a) as $\sqrt{(4S_a/\pi)}$ and substituting into (1) gives:

$$f = b/[\sqrt{(S_a)}] \qquad (2)$$

where b is constant and S_a is mirror surface area. Given this expected inverse relationship between C_f and mirror surface area, we inverse-transformed mirror surface area before adding it as a factor to the linear model. We included two interaction terms; one between phase and HCW, and the other between phase and mirror surface area.

For each recording, we counted the average number of wing-strokes per second and the average number of peg strikes against the plectrum within each wing stroke. Given that chirp rate in other decticines has been shown to be a linear function of temperature (eg. Walker 1974), we included temperature as a factor in separate GLMs testing whether chirp rate and average peg strikes per chirp differed between phases.

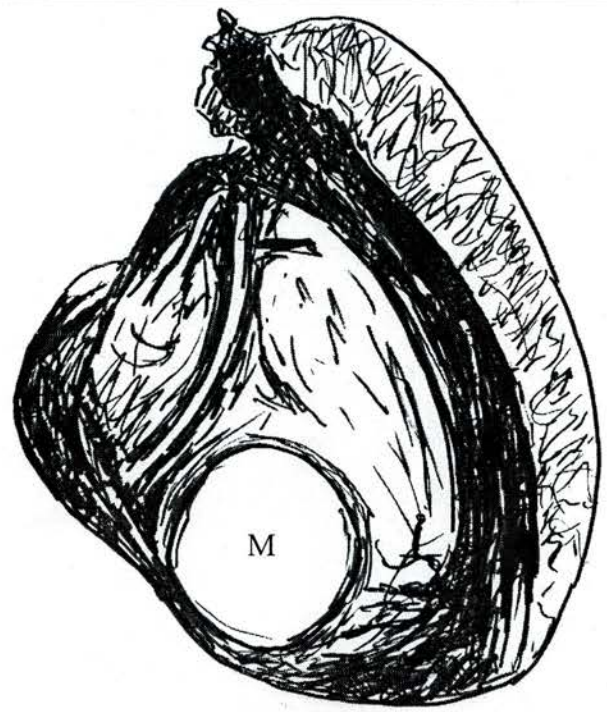


Figure 2: Dorsal view of a lower (right) elytra of *Anabrus simplex* showing the roughly circular mirror (M).

Morphology

We measured head capsule width (HCW), number of stridulatory pegs, the total surface area of the lower elytra and mirror surface area for all males. A small sample of 12 gregarious (LE) and 12 solitary (KF and IM) females were also measured for HCW to assess any sexual dimorphism, as described by Gwynne (1984), who found that females were larger than males in gregarious populations but not in solitary populations.

Results

Most of the variation (94.25%) in morphological and song traits was between phases ($F_{1,56} = 467.48$, $P < 0.001$) with only a small component (0.64%) amongst populations within phases (0.64%, $F_{3,56} = 2.45$, $P = 0.073$). We therefore pooled populations for further analyses.

Solitary and gregarious male Mormon crickets were completely distinguishable when morphological and song characteristics were considered together (figure 3). A discriminant

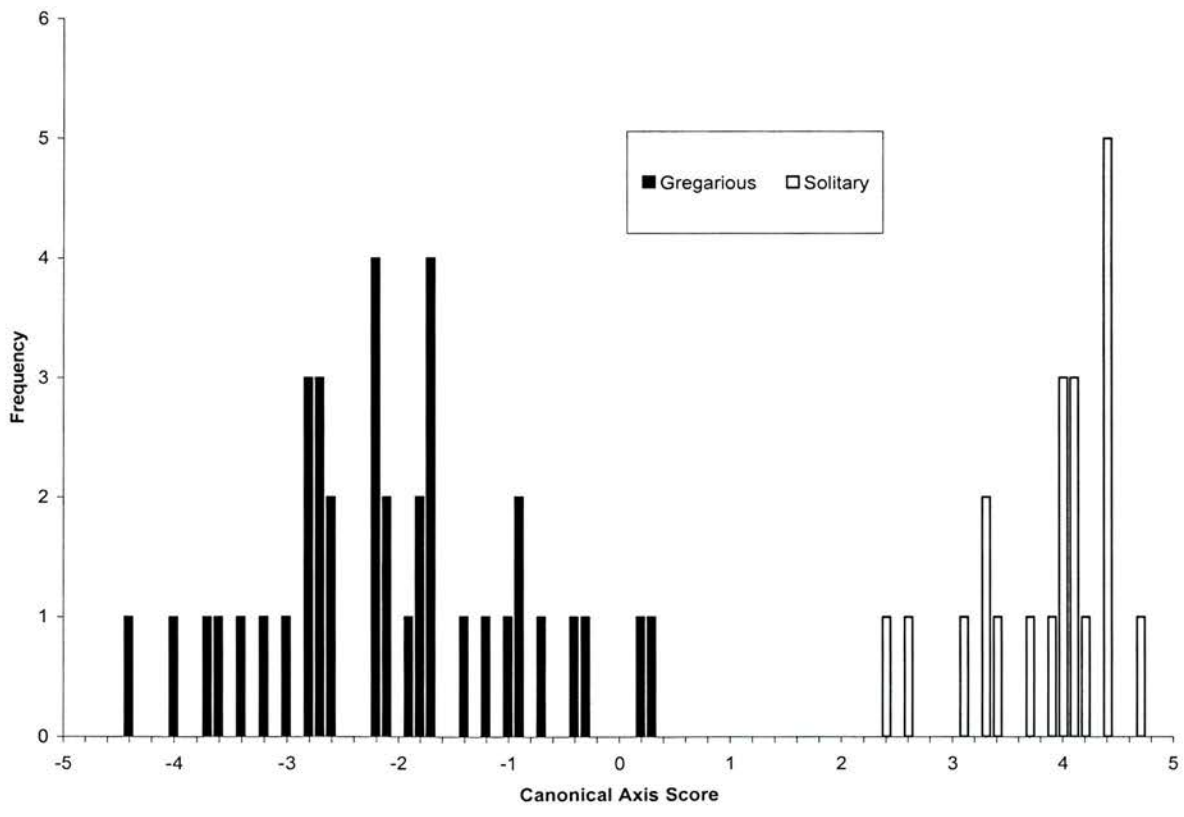


Figure 3: Solitary and gregarious Mormon crickets were completely distinguishable based on the first axis of a canonical variate analysis. As the canonical axis score increases, HCW, elytral surface area and mirror surface area decrease while chirp rate and peg strike rate increase.

analysis with cross-validation classified all samples into the correct group: solitary males had smaller HCWs (R-sq = -0.971, $p < 0.001$), smaller elytral surface area (R-sq = -0.862, $p < 0.001$), smaller mirror surface areas (R-sq = -0.367, $p = 0.004$), higher carrier frequencies (R-sq = 0.801, $p < 0.001$), chirped faster (R-sq = 0.889, $p < 0.001$) and had more peg strikes per wing stroke (R-sq = 0.538, $p < 0.001$). The only insignificant canonical variate correlation was the number of pegs on the stridulatory file (R-sq = -0.170, NS).

Reluctance to sing

Overall, gregarious males were more reluctant to sing (G-test: $G_1 = 4.38$, $P = 0.036$) (figure 4). Year was not significant as a main effect (G-test: $G_1 = 2.04$, NS), but there was a significant interaction between year and phase (G-test: $G_1 = 10.64$, $P < 0.05$). Gregarious males were more reluctant to sing in 2004, but in 2005 solitary males were more reluctant to sing.

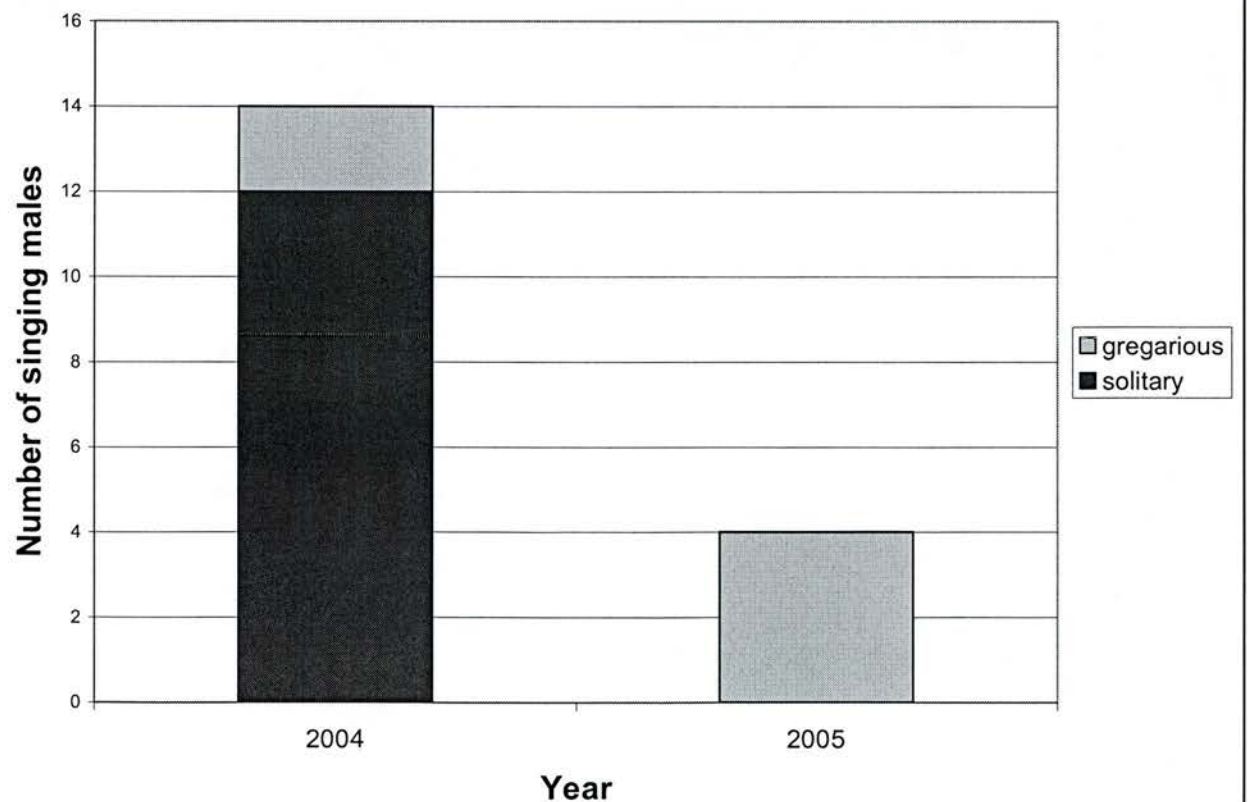


Figure 4: Gregarious males were more reluctant to sing overall, although they were less reluctant to sing in 2005. This is the reverse of the trend seen in 2004, and the cause of a significant interaction between year and phase (G-test: $G_1 = 10.64$, $P < 0.05$).

Sexual dimorphism

Females were larger than males (GLM: $F_{1,81} = 98.82$, $P < 0.001$) and gregarious individuals larger than solitary individuals (GLM: $F_{1,81} = 347.65$, $P < 0.001$), but there was no variation in sexual dimorphism between the phases (figure 5). Additionally, the variances in HCW both between phases and between sexes within phases did not differ (Bartlett's test: $F = 3.84$, NS).

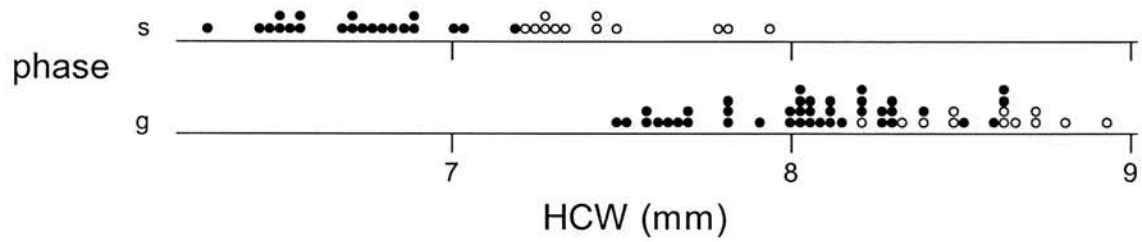


Figure 5: Head capsule width (HCW) in solitary and gregarious individuals studied. Open circle = female, solid circle = male.

Differences in song structure between phases

Temperature did not have an effect on chirp rate or the number of peg strikes per chirp, but solitary individuals chirped faster than gregarious ones (GLM: $F_{1,57} = 139.28$, $P < 0.001$) and used more stridulatory pegs for each wing stroke (GLM: $F_{1,57} = 20.77$, $P < 0.001$). Despite these

differences in song structure, the number of stridulatory pegs did not differ between solitary and gregarious males (t-test: $t_{59} = 1.24$, NS).

As expected, C_f varies negatively with size (HCW) and mirror surface area, but it also differs between phases. A full GLM showed that these three factors independently influenced C_f, suggesting that there were effects beyond the expected differences due to size (table 1). Most interestingly, the relationship between mirror surface area and C_f varied between the phases (figure 6, table 1), which is not predicted if C_f is solely determined by mirror surface area.

Table 1: Determinants of male carrier frequency (C_f)

Source	df	F	P
Phase	1,56	103.45	0.001
Mirror surface area	1,56	11.57	0.001
Head capsule width	1,56	5.24	0.026
Phase×Mirror surface area	1,56	5.18	0.027

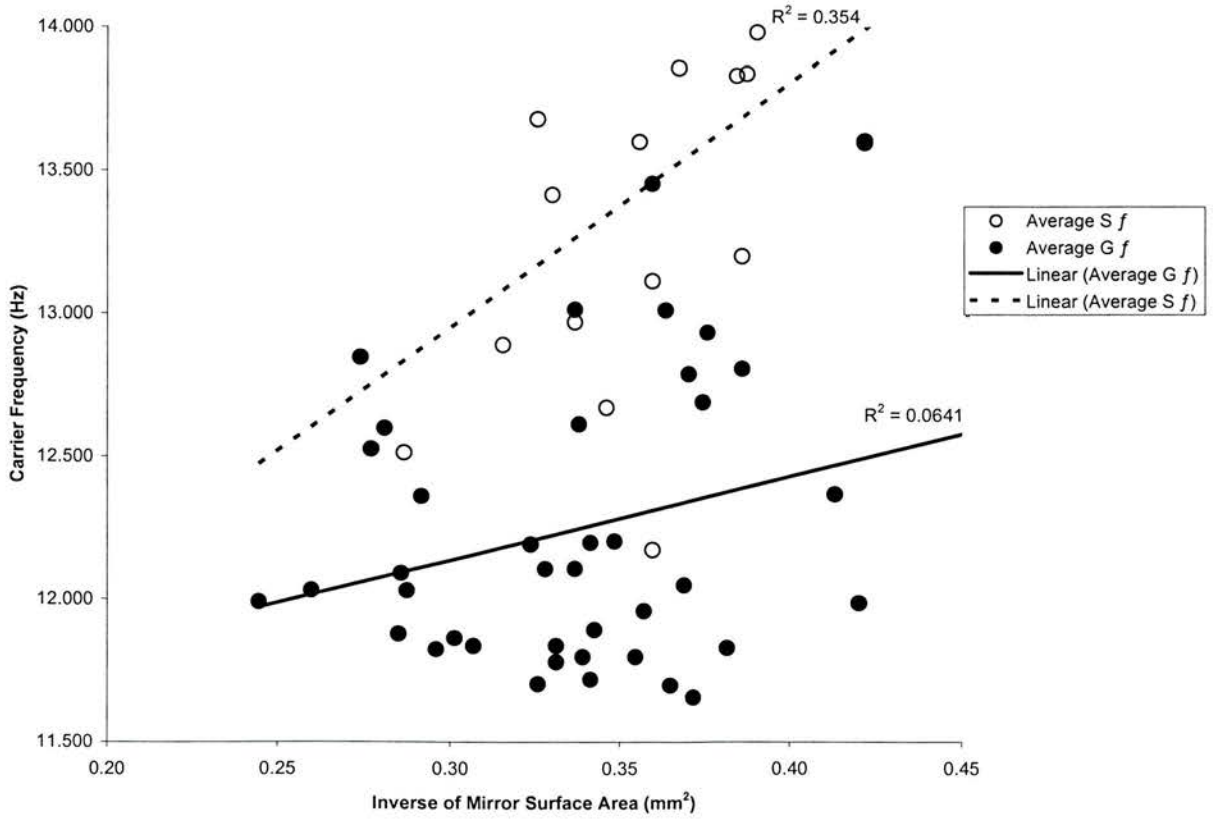


Figure 6: The slope of the regression of C_f on the inverse of mirror surface area was significantly steeper for solitary Mormon crickets.

Discussion

Differences between phases

Mormon crickets in this study showed significant variation in morphology, song structure and carrier frequency between gregarious and solitary phases. Compared to solitary crickets, gregarious individuals were larger, chirped slower, used fewer of their stridulatory pegs per chirp and their carrier frequency was lower. However, there were unexpected aspects to the song differences. Solitary males have a higher carrier frequency independent of mirror size, and the slope of the regression of carrier frequency on mirror size is steeper in solitary crickets. There are two levels of explanation—proximate (mechanistic) and ultimate (evolutionary)—that may account for this difference.

On a proximate level, the influence of factors that predict carrier frequency may differ between phases. A prediction of the clockwork model (developed in trilling species) is that physical properties of the mirror—for example size, shape and tension—predict carrier frequency (Elliott and Koch 1985). However, an alternative model developed from studies of the field cricket *Gryllus bimaculatus* suggests that the volume of the subalar space—a pocket of air enclosed by the raised tegmina during singing—acts as an acoustic resonator (Stephen and Hartley 1995). Field crickets may vary the volume of this space, and thus the carrier frequency of their calls, using a mechanism of auditory feedback control (Stephen and Hartley 1995). This study did not explicitly examine all the factors that may predict carrier frequency in Mormon crickets; however, the fact that the relationship between mirror size and frequency varies between phases suggests that there may be variation between phases in the density, tension or shape of the mirror or the subalar volume.

Other song parameters also varied between phases. Solitary and gregarious Mormon crickets did not differ in the number of pegs on their stridulatory files, but solitary crickets used significantly more pegs during each wing stroke and chirped faster. Thus during an average wing-stroke, solitary individuals struck a greater proportion of their stridulatory pegs on the plectrum.

What ultimately accounts for the morphological and song differences between phases? Gwynne's (1984) work concluded that sex role reversal caused sexual selection to be stronger on

females than males in gregarious populations. Sex role reversal has been shown to cause changes in song parameters in other Tettigoniid species in lab experiments (eg. Ritchie *et al.* 1998) and in the wild (eg. Gwynne *et al.* 1998), and gregarious Mormon crickets are likely to have experienced historically consistent sex role reversal (Gwynne 1984). Since carrier frequency and chirp rate can convey information about present and past condition (Scheuber *et al.* 2003a, 2003b) and variation in male song can be subject to strong selection (Andersson 1994, Henry 1994, Simmons 1995, Simmons and Ritchie 1996, Mendelson and Shaw 2005), it is likely that solitary and gregarious phases have experienced differing sexual selection pressures. However, solitary and gregarious Mormon crickets in the present study also represent discrete genetic lineages that split during the Pleistocene (Bailey *et al.* 2005). It is unclear whether differences in sexual selection pressure on song caused divergence in song traits between these allopatric populations (eg. Panhuis *et al.* 2001) or whether song differences have arisen via drift (eg. Roff *et al.* 1999).

Sexual size dimorphism

Gwynne (1984) described sexual size dimorphism (females larger than males) in gregarious, but not in solitary, populations of Mormon crickets, and attributed this to differences in sexual selection pressure resulting from sex role reversal. We have shown that in 2005, females are larger than males in both gregarious and solitary populations. The interaction between sexual size dimorphism and phase is insignificant, indicating that the direction of the difference is the same and of similar magnitude in both groups, and we also found no difference in the variances.

This finding would appear to refute Gwynne's (1984) prediction that females should be larger than males in role-reversed populations, and the similarity in variance between sexes and phases does not support our hypothesis that gregarious males have experienced relaxed sexual selection. These characters could be fixed and independent of OSR, indicating variation between subspecies instead. Natural selection or drift may have driven divergence in body size as well as the other characteristics we measured and sexual selection may have driven sexual size dimorphism within both phases.

Reluctance to sing

In this study, gregarious males were more reluctant to sing overall, but there appears to be temporal variation in this behaviour. Temporal or spatial variation in OSR (eg. Gwynne *et al.* 1998) could account for this, but reluctance to sing is also likely influenced by predation risk. Numerous studies of Ensiferan species have identified acoustically-orienting predators and parasites and Ensiferan adaptations for avoiding them (eg. Belwood and Morris 1987, Zuk *et al.* 1993, Römer and Bailey 1998, Schul and Patterson 2003). Parasitism risk has been shown to be a significant selection pressure on calling parameters in *Teleogryllus oceanicus*, with crickets in parasitized populations less likely to sing (Zuk *et al.* 1993), because parasitoid flies orient towards males with longer song parameters (Zuk *et al.* 1998). Mormon cricket predators are diverse and include arachnids, hymenopteran parasites and lizards (Sorenson and Jeppson 1940), though most are likely to be rodents or birds against which gregarious bands give protection (Sword *et al.* 2005). Temporally variable sexual selection pressure on male song may be influenced by OSR; however, predation risk may provide natural selection pressure on male song depending on the abundance of predators at any given time and whether the crickets are vulnerable to predators in solitary populations, or protected in a gregarious band.

Conclusions

The behavioural and morphological differences we have found between solitary and gregarious Mormon cricket populations add to the previous discovery of a genetic division that implies a distinct evolutionary history for around 2 M years. Some of these are expected given the predictions based on life history and OSR differences between phases. The unusual variation in the relationship between mirror size and carrier frequency is more surprising, however, and given the potential influences of the physical properties of the mirror or volume of subalar space on carrier frequency, the variation in Mormon crickets provides an opportunity for further research into the different models of sound production. Such research has hitherto bypassed non-trilling, but stridulating, species (though see Montealegre-Z and Mason 2005).

One definition of subspecies is that they should show "concordant distributions of multiple, independent, genetically based traits" (Avise and Ball 1990). It is beyond the scope of

this study to disentangle the potential phylogenetic effects on the traits studied with the potential effects of OSR and sex role reversal, but the phases of Mormon crickets satisfy this criterion. Further studies should address extent to which these differences are fixed, and their influence on reproductive isolation.

Acknowledgements

We gratefully thank the following organizations, which contributed funding towards this project: The Linnean Society, The British Ecological Society, The Russell Trust (St. Andrews) and The Orthopterists' Society. Gordon S. Brown provided assistance with digital imaging and morphological measurements, and Ellen M. Bailey lent her skills in the production of Mormon cricket cages.

Beyond The Point Of No Return? A Comparison Of Genetic Diversity In Captive And Wild Populations Of Two Nearly Extinct Species Of Goodeid Fish Reveals That One Is Inbred In The Wild[3]

NW Bailey, C Macías Garcia, MG Ritchie

Abstract

The relative importance of genetic and non-genetic factors in extinction liability has been extensively debated. Here we examine the levels of genetic variability at thirteen (seven informative) microsatellite loci in wild and captive populations of two endangered species of Mexican Goodeid fish, *Ameca splendens* and *Zoogoneticus tequila*. Allelic diversity was higher in the wild populations, and F_{IS} lower. Values of Θ ($=4N_e\mu$) were estimated using a coalescent approach. These implied that the effective population size of all captive populations of *Ameca splendens* were smaller than that of the wild population. However, the wild population of *Z. tequila* does not show a significantly greater estimate of Θ. We used the Beaumont (1999) approach to infer population declines, and found that both species showed clear evidence of a decline, though this was stronger and probably occurred over a longer period of time in *Z. tequila* than *A. splendens*. We conclude that *Z. tequila* shows clear evidence of a decline in effective population size, probably before the captive populations were established. We discuss implications for the conservation of critically endangered populations.

[3] Together, CMG and MGR did most of the writing in the introduction and discussion. I have also added my input to these sections.

Introduction

The relative importance of genetic and non-genetic factors in extinction liability has been extensively debated since Lande's (1988) landmark paper suggested that demographic factors, such as disturbance-induced life history changes or habitat destruction, were more important predictors of extinction than inbreeding. A recent meta-analysis by Spielman *et al* (2004) has shown that critically endangered species usually have reduced genetic variability relative to non-threatened sister species. This has been interpreted as giving renewed support to the idea that genetic factors are important (deSalle 2005), although clearly the two (demographic and genetic changes) must be intimately related.

It seems probable that genetic variability is important to the restoration of extinct or near-extinct species into the wild. More genetically heterogeneous stocks are likely to have a better chance of establishing themselves in an environment which may be different in some respects to the original environment, though quantitative data on this are lacking. Ark projects should be managed to maintain genetic variability in stocks of endangered species, and considerable effort has been put into this. However, it is likely that stocks of species which are being maintained for potential reintroduction will previously have been subject to a level of inbreeding in the natural habitat, either due to natural expansion-contraction cycles or more recent anthropogenic factors prior to their maintenance in captivity. The influence of historical bottlenecks on effective population size and genetic variability can usually only be inferred indirectly. Whilst population bottlenecks must be important in some well-known cases of inbred natural populations (Merola 1994, Crnokrak and Roff 1998), other studies imply that even severe historical bottlenecks can sometimes have remarkably little effect on reductions in genetic variation at neutral markers (Groombridge *et al.* 2000, Nichols *et al.* 2001) and quantitative genetic variation for fitness traits (Howard 1993, Saccheri *et al.* 1996), two measures of genetic depletion which need not be correlated (Britten 1996).

Goodeid fish have often been reported as extinct or extirpated from the wild (De la Vega Salazar *et al.* 2003a). This is a family of North American Cyprinodontid topminnows with a continental distribution in areas of increased geological and historical desertification (Webb *et al.* 2004). One species (*Empetrichthys merriami*) in the sub-family Empetrichthyinae (consisting of the genera *Crenichthys* and *Empetrichthys,* originally found in Nevada, USA) became extinct

in the last century, and two are classified as vulnerable (IUCN red book 2000). The more numerous and ecologically diversified sub-family, the viviparous Goodeinae, occupies most basins of Central Mexico. Of the 37 recognised species two are reported extinct (*Allotoca meeki* and *A. catarinae*, in addition *Characodon garmani* was described from a single specimen), one is extirpated from the wild (*Skiffia francesae*) and two have been found in small natural populations despite being repeatedly reported as extinct (De la Vega Salazar *et al.* 2003b). *Ameca splendens* and *Zoogoneticus tequila* are native to the Teuchitlán River, in the Ameca basin (western Mexico), which has experienced massive changes following the building of a dam in 1955. Captive populations of both species have been kept by zoological institutions and dedicated aquarists, and it was from such populations and museum specimens that *Z. tequila* was described (Webb and Miller 1998). A recently discovered wild population of *Z. tequila* is composed of only a handful of adult fish and a few tens of juveniles (De la Vega Salazar *et al.* 2003a, b), whereas relatively large populations of *A. splendens* are found in spa springs (De la Vega Salazar *et al.* 2003a). Here we compare the genetic variability, using microsatellite loci, of wild samples of *Z. tequila* (of adults from springs in the Teuchitlán area and their offspring) and *A. splendens* with samples from aquarists stocks maintained in the UK for up to 30 years (figure 1).

Materials and Methods

Sampling

Tail fin clips were taken from four populations of *Ameca splendens* and three populations of *Zoogoneticus tequila*. Information about populations sampled is given in Table 1. Briefly, one wild population of *A. splendens* was sampled from its native range in the headwaters of the Teuchitlán river, and samples were obtained from three captive aquaria populations in the UK. The recently discovered wild population (also in the headwaters of the Teuchitlán river) of *Z. tequila* was sampled. This is the only known wild sample of *Z. tequila.* To increase the number of wild alleles sampled without further interference with the population, we included the F_1 offspring of the original population (it was expected that some females collected from the wild were carrying young from previous matings; see results). Fin clips from a captive population of *Z. tequila* were also obtained from Chester Zoo (and both sexes were represented).

Figure 1: Aquaria specimens of *Zoogoneticus tequila* (left) and *Ameca splendens* (right).

Table 1: Populations of *A. splendens* and *Z. tequila* sampled.

Population	*Number*	*wild/captive*	*Origin*	*Notes*
Z. tequila				
Mexico Parents	13	wild	Teuchitlán	Parental generation collected from the wild
Mexico Offspring	18	wild	Teuchitlán	F_1 generation of above, reared in aquaria
Chester Zoo	20	captive	Chester Zoo	Zoo population established with individuals from Bolton Museum (n=4) in 1995.
A. splendens				
Mexico	20	wild	Teuchitlán	
St. Andrews	20	captive	St. Andrews Aquarium	Population established with individuals (n=16) from London Zoo in 1997. London Zoo population originally started in 1961 using wild individuals (n=12) from Mexico.
Chester Zoo	30	captive	Chester Zoo Aquarium	Population comprised of samples from Bolton Museum (n=7), London Zoo (n=5) and Bristol Zoo (n=4) established in 1995.
CZJG	20	captive	Chester Zoo Aquarium	Population founded from a 48-individual subset of the original Chester Zoo population in 2001.

DNA extraction and microsatellite analysis

Genomic DNA was extracted from ethanol-preserved fin clips using the PureGene protocol (Gentra Systems, Minneapolis, MN). We tested eight microsatellites that had been previously developed from various Goodeid species (Hamill *et al.* submitted): As2, As5, Ca6, Ca10, Iw193, Iw196, Xc18 and Xc25, and a further five from the literature: Zt1.2, Zt1.3, Zt1.6, Zt1.7 and Zt1.9 (Boto & Doadrio 2003). Each 15 μL PCR reaction contained 0.2 mM each dNTP, 0.20-0.33 μM each fluorescently labeled primer (Proligo), 1.5 μL 10X NH_4 reaction buffer, 1.5 mM $MgCl_2$, 0.1 μL Biotaq DNA polymerase (Bioline), and 10-300 ng DNA template. The PCR profile for all reactions was: (1) 5 min at 94°C initial denaturation, (2) 15 s at 94°C denaturation, (3) 10s at 60°C annealing (55°C for Ca10 and 62°C for Ca6), (4) 1 min at 72°C extension, (5) 39 repeats of steps 2 through 4, and (6) 10 min at 72°C final extension. PCR

products were checked on 1.5 % agarose gels. All samples were analysed on a Beckman Coulter 3000 automated sequencer using 0.5-2.0 μL PCR product plus 0.5-1.0 μL 400 or 600 bp DNA ladder (Beckman-Coulter).

Four loci, Ca6, Ca10, Iw193, and Xc25 were monomorphic in both species and were not included in the analysis. Loci Zt1.3 and Zt1.7 did not amplify cleanly despite attempts to re-optimise, so they were also excluded. Locus Zt1.2 was monomorphic in *Ameca splendens* but polymorphic in *Z. tequila*.

Data analysis

Hardy-Weinberg equilibrium and pairwise population differentiation were tested using Genepop v.3.4 (Raymond and Rousset 1995). Within population measures of mean heterozygosity and mean number of alleles per locus are often presented as proxies for genetic diversity; however, we estimated allelic richness to correct for bias due to sample size. We also calculated F_{IS} for each population at each locus, and for each population across all loci combined, to compare levels of inbreeding. Allelic richness and F_{IS} were calculated with FSTAT v.2.9.3 (Goudet 2001).

We used Migrate v.1.7.3 (Beerli 2002), part of the package LAMARC, to obtain maximum likelihood estimates of Ne of each population of *A. splendens* and *Z. tequila*. This uses a coalescent approach coupled with a Monte-Carlo Markov Chain technique to estimate Θ ($\Theta = 4Ne\mu$, where Ne is effective population size and μ is the mutation rate) for each population. Migrate uses a random seed to initiate calculations, so replicate estimates of Θ vary. Ten trials were performed for each species and results compared using one-way ANOVAs.

The above analysis is useful for estimating contemporary effective population sizes, but is limited because it cannot detect whether a population has been historically declining, expanding or stable. The programme msvar (Beaumont 1999) is also based on a MCMC technique, but uses a coalescent approach (assuming a step-wise mutation process) to provide joint likelihood estimates for two additional population demographic parameters of interest to this study. The first is r, where $r = N_0/N_1$, where N_0 is the current size of the sampled population and N_1 is the size of a single stable population before decline or expansion. A declining population is expected to give $\log_{10}(r) < 0$ and an expanding one $\log_{10}(r) > 0$. A stable population size would give $\log_{10}(r) = 0$. The second parameter is t_f, which is the time (in

generations) since the stable ancestral population began to decline or expand (t_a), scaled by the current population size (N_0), giving $t_f = t_a/N_0$. We estimated the parameters of interest for *A. splendens* and *Z. tequila* based on field observations and historical data (Macías Garcia pers. comm.). For *A. splendens*: $N_0 \approx 4{,}000$, $N_1 \approx 10{,}000$ and $t_a \approx 50$. For *Z. tequila*: $N_0 \approx 90$, $N_1 \approx 4{,}500$ and $t_a \approx 50$.

We hypothesized that the wild populations of *A. splendens* and *Z. tequila* have experienced recent population declines and therefore used an exponentially varying population model in the analysis. Only wild populations from each species were included in this analysis, and the data from Mexico Parents and Mexico Offspring in *Z. tequila* were pooled as a single wild population. Since the MCMC chain may require a period of burn-in, we discarded the first 10,000 update steps out of 10^9 thinned update steps. Additionally, starting seeds and scale values were varied for three independent runs per species to check for convergence. Our parameter bounds on $\log_{10}(r)$ and $\log_{10}(t_f)$ were (-4-0) and (-2-1), respectively. Increasing the bounds beyond these reduced convergence, but we limited the main bounds used to our biologically-derived estimates. Similar bounds were used by Beaumont (1999), and encompass a range of parameter values that we considered to be biologically sensible given the estimates of N_0, N_1 and t_a for both species. For example for r, N_0 was allowed to be—at the lower bound—four orders of magnitude smaller than N_1, and—at the upper bound—equal in size to N_1. We plotted the joint posterior distribution of $\log_{10}(r)$ and $\log_{10}(t_f)$ following the procedure of Beaumont (1999) and Storz and Beaumont (2002).

Results

Hardy-Weinberg equilibrium and population differentiation

All *Z. tequila* populations were in HWE at each locus and across all loci combined after Bonferroni correction. In *A. splendens* locus As5 was out of HWE in all populations, indicating potential presence of null alleles, and was therefore discarded from all subsequent analyses. Following this, all *A. splendens* populations were in HWE across the remaining five loci after Bonferroni correction.

Pairwise tests of differentiation showed that all *Z. tequila* populations differed significantly in allele frequency, except Mexico Parents and Mexico Offspring (χ^2_{14}=11.056,

NS), and the only *A. splendens* populations that did not differ significantly were Chester Zoo and Chester Zoo Jaguar House (χ^2_8=11.086, NS).

F_{IS}

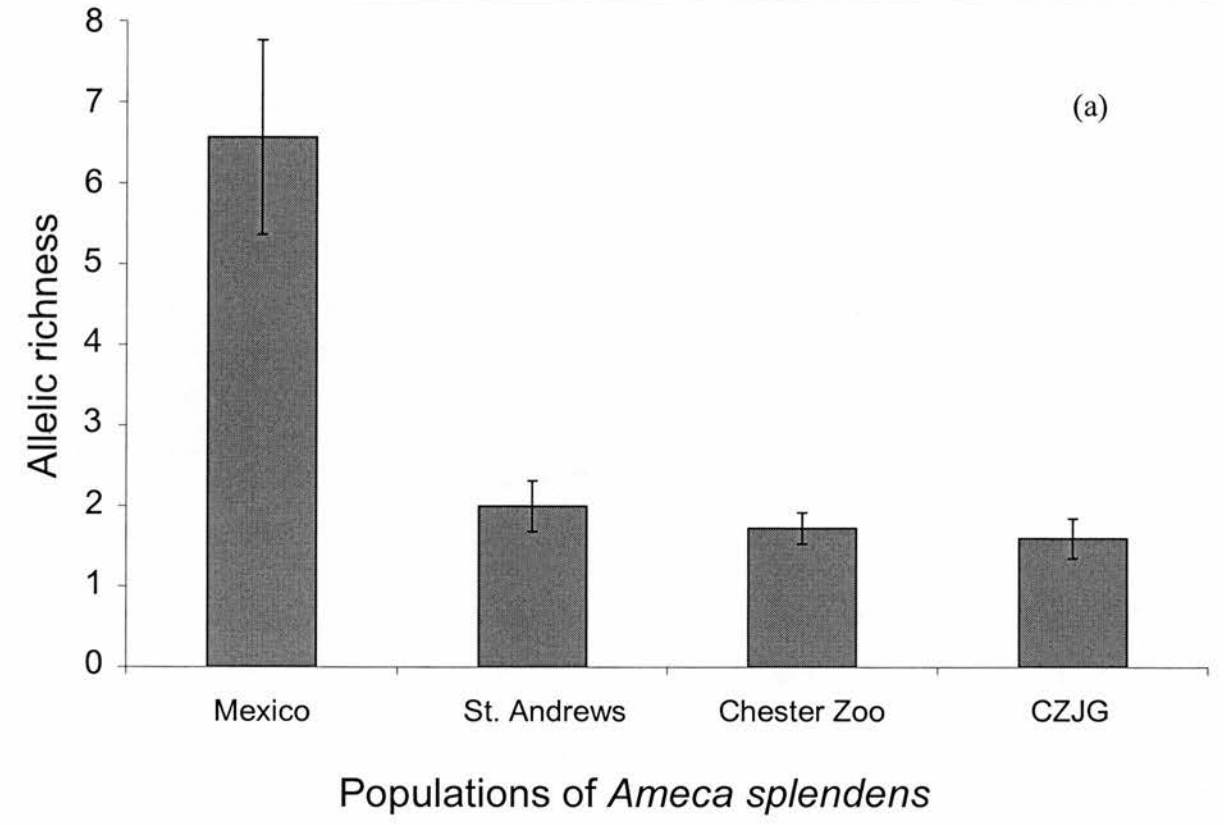
Table 2 shows F_{IS} values for all populations at each locus, and across all loci (excluding As5 in *A. splendens*). Larger values are usually seen only in the captive populations, indicating inbreeding (heterozygous deficit).

Table 2: F_{IS} values for each population (and locus) of *A. splendens* and *Z. tequila*, and Θ values per population. Zt1.2 was not analysed in *A. splendens* because it is monomorphic in this species; "na" indicates that a population was monomorphic at that locus. * As5 was discarded from all analyses involving *A. splendens* because it was out of HWE in all populations. Θ 95% CI = ± 0.102702 (*Z. tequila*) and 95% CI = ± 0.09495 (*A. splendens*) using pooled s.d. from all populations within a species.

	Z. tequila						
	Mexico			*A. splendens*			
Locus	Parents	Offspring	Ch. Zoo	Mexico	St. Andrews	Ch. Zoo	CZJG
As2	-0.114	0.022	-0.192	-0.178	0.016	0.039	0.587
Iw196	-0.059	-0.162	na	0.236	0.163	0.016	0.191
Xc18	0.27	-0.146	-0.029	-0.002	0.706	0	na
Zt1.6	-0.3	-0.311	0.333	-0.086	na	na	na
Zt1.9	0.009	-0.064	0.294	-0.074	0.227	0.133	-0.393
Zt1.2	0.008	-0.136	na				
As5	0.063	-0.483	0.234				
All loci	-0.014	-0.164	0.125	-0.019	0.282	0.054	0.081
Θ	0.66	0.68	0.82	1.95	0.49	0.53	0.47

Genetic diversity

In both *A. splendens* and *Z. tequila*, allelic richness was significantly higher in the wild Mexican populations than in the captive populations (figures 2 a and b, respectively). Other diversity indices such as mean heterozygosity, mean number of alleles per locus and total number of alleles per locus showed similar patterns.



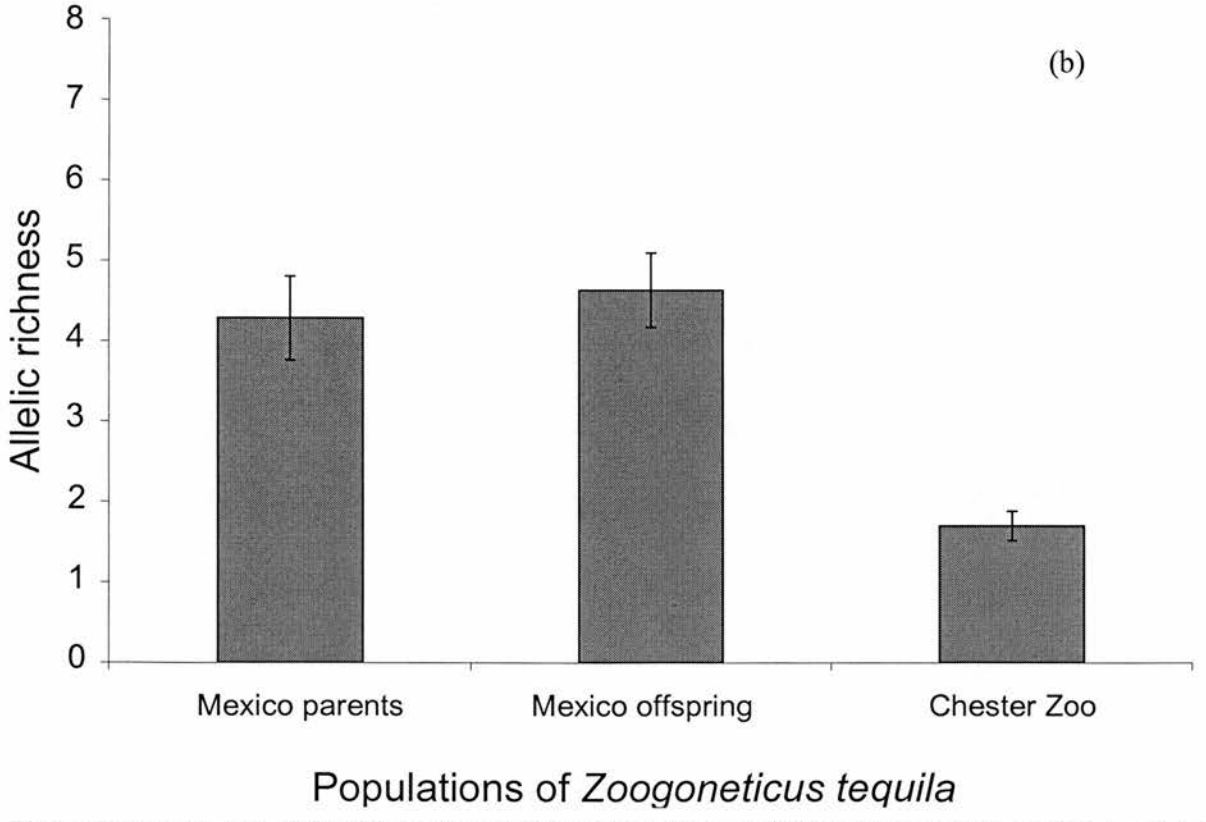


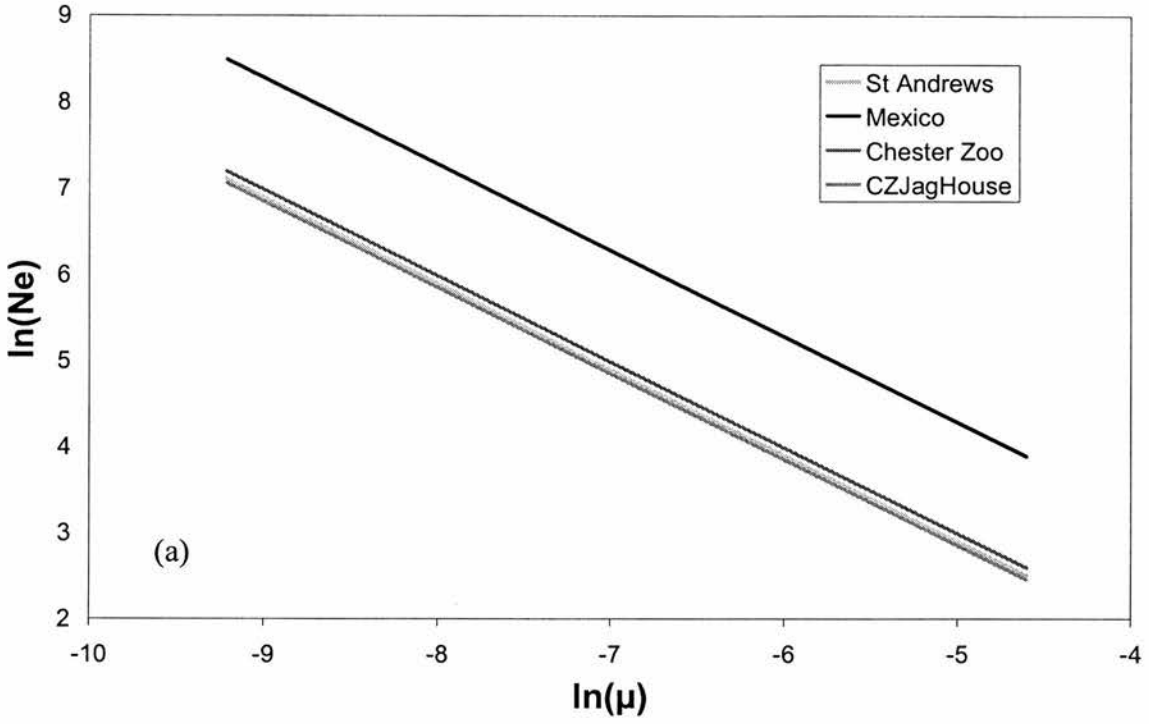
Figure 2: Mean (± s.e.) allelic richness is significantly higher in the wild Mexican populations of **a)** *A. splendens* (Kruskal-Wallis: $H_{3,16}$=9.66, p<0.02) and **b)** *Z. tequila* (Kruskal-Wallis: $H_{2,18}$=12.69, p=0.002).

Theta (Θ) estimates

In *A. splendens,* mean Θ across 10 trials was significantly higher in the wild Mexican population (by a factor of four) than in the captive populations (table 2) (one-way ANOVA: $F_{3,36}$=224.51, p<0.001, Tukey's comparison significant at 3.81). However, in *Z. tequila*, there was no significant difference between the mean estimates of Θ of the wild and captive populations (table 2) (one-way ANOVA: $F_{2,27}$=2.85, p=0.075). The mean Θ values of *Z. tequila* were similar to those found in the captive *A. splendens* populations.

Comparison of Ne over a range of mutation rates (μ)

The central aim of our analysis was to draw a comparison of genetic variation between wild and captive populations; however, these data do allow an inference of relative effective population size. Any such estimate relies on knowledge of the mutation rate (μ) of the genetic markers used in the analysis. The analysis performed in Migrate assumes a constant—and equal—mutation rate across all microsatellite loci, but estimates of microsatellite mutation rates range widely depending on both the organism and the locus examined (DeWoody and Avise 2000, Schlötterer 2000, Schug *et al.* 1998, Weber and Wong 1993). DeWoody and Avise (2000) used microsatellite mutation rates between 10^{-2} and 10^{-4} (based on studies in humans and *Drosophila melanogaster*) to estimate Ne in freshwater fish. Rather than derive one estimate, here we plot average Ne against a range of mutation rates for *A. splendens* and *Z. tequila* (figures 3a and 3b, respectively). As with the Θ estimates, average Ne of the wild *A. splendens* population is highest, varying from 19 to 4867 in the selected range of mutation rates. Average Ne of both wild and captive *Z. tequila* populations are similar to that of the captive *A. splendens,* in the lowest instance (CZJH) only reaching 1164 (for a mutation rate of 10^{-4}; figure 3).



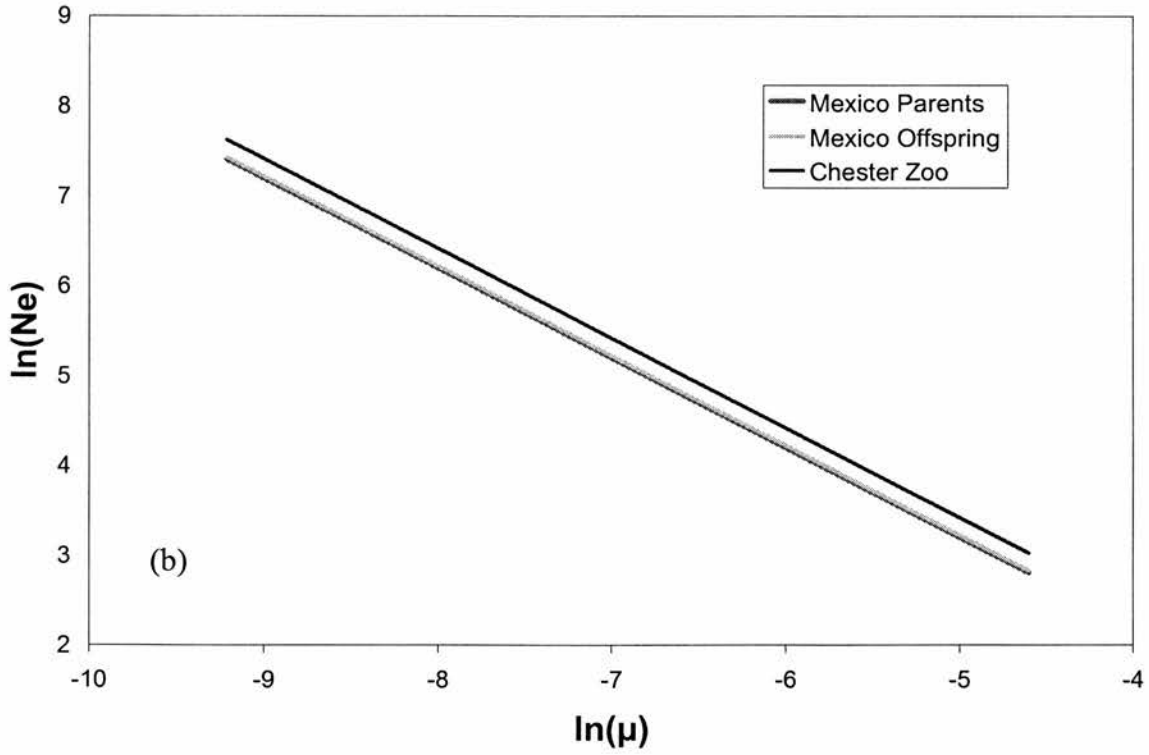


Figure 3: Relationship between ln of the average Ne for each population and ln of mutation rate (μ). **(a)** *A. splendens* , **(b)** *Z. tequila*.

Detecting population decline

Both *A. splendens* and *Z. tequila* show evidence of exponential population decline (figure 4), although the evidence in *Z. tequila* is stronger. The more severe decline in *Z. tequila* may have occurred over a longer period of time, as the 10% HPD contour for t_f is about one and a half orders of magnitude greater than that of *A. splendens*, indicating that the time since decline for *A. splendens* may have been much shorter. Contours for the highest posterior density (HPD) limits indicate the region within which the probability of the parameter values is a critical value (Beaumont 1999). For example, the 90% HPD contour gives the region within which the densest 90% of sample points lie, and can provide a measure of confidence in the parameter estimation. Convergence (i.e. consistency in the 90% HPD contours) occurred in both species over multiple long runs of 10^9 thinned update steps using variable starting seeds and parameter scale values. However, the presence of multiple 10% HPD peaks in each run of *A. splendens* suggests that convergence in this species may be weaker (see figure 4). The 90% HPD limits on $\log_{10}(r)$ never cross zero for either *A. splendens* or *Z. tequila* in any of the runs, however, which provides consistent support for exponential population decline in both species.

The stronger convergence in *Z. tequila*, i.e. the consistency of the 10% HPD contour across several runs, suggests that the population size of this species before decline was roughly 3 orders of magnitude larger than the present size. Convergence of 10% HPD contours in *A. splendens* is weaker; however, if we consider a range of values corresponding to the two peaks in figure 4 (which were also consistent across runs), a pre-decline population could have been between 1.5 and 3 orders of magnitude larger than the present size.

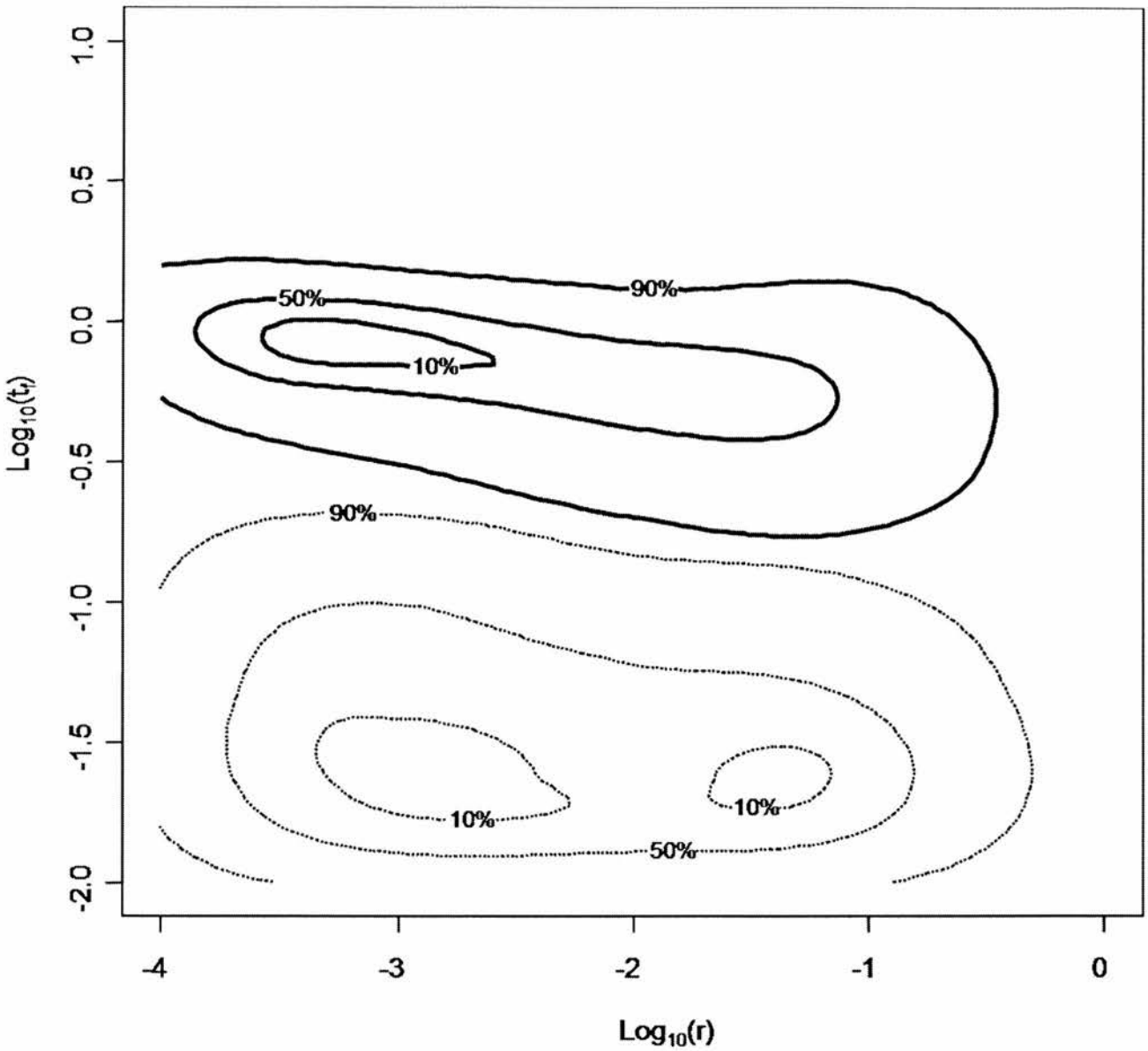


Figure 4: Joint posterior distribution of $\log_{10}(r)$ and $\log_{10}(t_f)$ for wild *A. splendens* population (dotted line) and wild *Z. tequila* population (solid line) showing 10%, 50% and 90% highest probability density (HPD) contours.

Discussion

We have found that a wild population of *A. splendens* is much more genetically diverse than stocks which have been maintained in captivity for up to ten years. Its effective population size is more than an order of magnitude greater. However, *Zoogoneticus tequila* (endemic to the basin of the small Teuchitlán River) provides a striking contrast. Although a wild population contains more alleles than the captive stock, the calculated Θ and therefore effective population

size is not significantly greater than that of the captive population. The allelic richness of the wild *Z. tequila* population is lower (around half) than that of wild *A. splendens*, and across the family Goodeidae as a whole allelic richness is more like that seen in wild *A. splendens* or greater (Hamill *et al.* submitted). The variability of allele lengths in *Z. tequila* suggests that the population decline has been stronger and over a longer period than that in *A splendens*, which is consistent with their current conservation status. Our interpretation is that the wild *Z. tequila* population, which is the only known extant population (De la Vega Salazar *et al.* 2003a, b), shows clear signs of declining genetic variability over its probable ancestral levels of diversity. A previous report using fewer markers (Boto and Doadrio 2003) found that *Z. tequila* had a lower heterozygosity than its common and widespread sister species (*Z. quitzeoensis*). Lande (1988) argued that demographic factors are paramount in driving extinction, a view supported by some recent reports of substantial demographic recovery of genetically impoverished populations (e.g. the Oryx, *Oryx leucoryx*; Mésochina *et al.* 2003; the elephant seal, *Mirounga angustirostrus*; Hoelzel *et al.* 1993), or others where population reduction was not clearly associated with a decrease in genetic variability (the Mauritius kestrel, *Falco punctatus*; Groombridge *et al.* 2000). It is even uncertain whether small population size and isolation necessarily lead to the loss of genetic diversity (e.g. the Mauritius kestrel, *Falco punctatus*; Groombridge *et al.* 2000 and the butterfly *Erebia epiphron silesiana*; Schmitt *et al.* 2005). It appears that sometimes, if the impact of anthropogenic factors is severe, populations may go extinct without undergoing genetic bottlenecks, and contrastingly that seemingly genetically impoverished populations can thrive, perhaps because inbreeding measured from neutral markers is often only loosely (if at all) related to quantitative fitness-related traits (Britten 1996, Butlin and Tregenza 1998, Slate *et al.* 2004).

Although it is extremely unlikely that all species become extinct due to inbreeding, the loss of genetic diversity can be a risk factor, and maintaining diversity is a major aim of ark projects. Estimates of the effect of inbreeding on fitness based on experimental manipulations (for instance the superiority of cross- over self-pollinated *Silene douglasii*; Kephart 2004) may not be applicable to naturally endangered species, which might have an evolutionary history of coping with low heterozygosity. Indeed, outcrossing may be detrimental for naturally inbred populations (e.g. outcrossed inbred males produce malformed tadpoles in *Rana temporaria*; Sagvik *et al.* 2004), particularly when they are outcrossed with captive populations, as demonstrated by the loss, rather than gain of genetic diversity in the Western mosquito fish,

Gambusia affinis following the release of fish from captive stocks (Stockwell *et al.* 1996). There is also evidence from captive studies that establishing populations from multiple stocks can lead to outbreeding depression in the related *G. holbrooki* (Leberg 1993).

There are no estimates of demographic parameters such as mean litter size, age at first reproduction or growth rate for either *A. splendens* or *Z. tequila*, yet recently established stocks derived from these wild parents are currently thriving in outdoor ponds. Thus there is no obvious evidence of inbreeding depression. We propose that, evidence of low Θ values for *Z. tequila* notwithstanding, the viability of wild stocks should not be compromised by attempts to increase their genetic diversity in the absence of evidence of inbreeding depression. In the case of *A. splendens*, introducing fish from captive stocks cannot substantially increase genetic diversity, and may promote the spread of deleterious alleles adapted to captivity (Lynch and O'Hely 2001). Indeed, there is evidence (Kelley 2002) that captivity has lead to a higher, potentially maladaptive aggressive behaviour in this species. The case of *Z. tequila* is more complex. Both captive and wild populations have private alleles and thus can potentially enrich the genetic diversity of each other. However, the wild population has survived in isolation, at small numbers, for at least 15 years (De la Vega Salazar *et al.* 2003b), which is probably long enough to have undergone local adaptation. In this case we face a poignant trade-off: to risk breaking down a locally adapted genome (and one purged of deleterious alleles) by introducing alleles adapted to captivity, or to risk local extinction due to stochastic environmental changes to which this genetically impoverished population cannot adapt.

The solution to such trade-offs partly depends upon our definition of a population. If we accept that the small deme of *Z. tequila* still holding out at a tiny spring in Teuchitlán is unique, we would be endorsing the value of its genetic identity. This would preclude rescue attempts such as that conducted with the population of adders (*Vipera berus*) at Smygehuk, Sweden (Madsen *et al.* 1999), where population decline prompted an exercise in genetic rescue which lead to a demographic recovery, but also produced a population that is genotypically different from the original one. If the objective was to save the unique population of adders at Smygehuk, the attempt failed, but if the aim was to keep alive a population of adders at Smygehuk (with some of its original genes), it was an unqualified success. We think that the unique population of *Z. tequila* at Teuchitlán is beyond the point of no return unless we manage to recover a substantial portion of its original habitat and thus allow it to increase its numbers. But we can

preserve some of its genes, we can combine them with those of the captive stocks, and we can design management programmes that minimise the negative impact of captivity by incorporating selective agents as similar as possible to those experienced in the wild. But, in the long run, husbandry leads to domestication. *Skiffia francesae*, a relative of these species, is completely extinct in the wild and we can detect no allelic variation at these loci in captive populations (pers. obs.; see also Boto and Doadrio 2003). Ultimately we must restore the environment to maintain critically endangered populations of species. In the mean time, efforts should be made to understand the relationship between quantitative fitness traits and inbreeding at neutral loci, so as to be able to assess the potential for survival of small populations when released in recovered environments.

Acknowledgements

We thank Gordon Reid, Mike Crumpler and Stephanie Sanderson (Chester Zoo), the staff at the St. Andrews Sea Life Centre, and Marina de la Vega-Salzar, Edgar Avila Luna and Mylene Mariette (UNAM) for providing samples. Shane A. Webb, Tanya Hamill and Ruth Hamill helped with the development of microsatellites, and J. Graves and Anne E. Magurran kindly read and commented the manuscript, and Mark A. Beaumont advised on the analyses of population decline. This research adhered to the Association for the Study of Animal Behaviour/ Animal Behavior Society Guidelines for the Use of Animals in Research.

GENERAL CONCLUSION

Synopsis of Mormon cricket research

Hewitt (2000) identified a need to develop more species phylogeographies to better establish the general principles of regional historical phylogeography. One distinction to be made in phylogeographic studies, however, is the likely timescale of the genetic differentiation being examined. It is possible to partition events having an effect on genetic structure during the evolutionary history of a population or species into early or "deep" versus recent or "shallow" levels (Riddle 1996). The former are more likely to be uncovered using mtDNA studies (Avise *et al.* 1987) whereas phylogenetic studies using DNA fingerprinting methods (eg. AFLPs) or microsatellites may elucidate more recent events (Bossart and Prowell 1998, Knowles and Richards 2005). In the present studies of Mormon crickets, population genetic structure due to such recent events is superimposed on a deep mtDNA division corresponding to east-west refugial phylogroups, and we have been able to distinguish biotic and abiotic factors that predict these different levels of genetic structure.

The population genetic patterns in Mormon crickets favour the hypothesis that vicariant events during the Pleistocene separated populations into eastern and western refugia, and life history differences between solitary and gregarious Mormon crickets—namely dispersal ability—predict differences in population genetic structure between the clades. Variation in dispersal ability between the refugial clades may have arisen as a result of selection for differing responses to climatic events during Holocene recolonisation. The dominant paradigm in population genetics is that dispersal is expected to act as a homogenizing force on population genetic structure by erasing any differentiation that may accumulate due to drift or selection (Lowe *et al* 2004), although this has been challenged by recent studies in birds showing that non-random dispersal can produce genetic differentiation (see Coltman 2005). Nevertheless, such a homogenizing effect on population genetic structure has been observed in another North American Orthopteran species with migratory tendencies, *Melanoplus sanguinipes* (Chapco *et al.* 1992 and 1994). What the present studies cannot distinguish, however, is the degree to which these differences have persisted in western versus eastern clades of Mormon crickets.

The answer to this question largely depends on the degree to which solitary and gregarious phenotypes are plastic. If these phenotypes are not fixed, it must also be determined what cues are responsible for the transition from one phase to the other, and whether the transition threshold is the same in both eastern and western clades or if, as has been found in the European earwig *Forficula auricularia*, transition thresholds (or "switchpoints") between alternative morphologies differ between populations (Tomkins and Brown 2004). Recently, Sword (2005) found that amongst Mormon crickets collected in Utah that hatched from eggs deposited by a gregarious band the previous year, behavioural phase changes were not density-dependent. Instead, inter-individual interactions had a greater influence on migratory behaviour.

It is important to emphasize that our genetic work does not reject the idea that the solitary/gregarious distinction is phenotypically plastic; indeed, the solitary western population at Little Brush Creek (LBC) in Utah, which is genetically indistinguishable from the rest of the western populations, provides a critical exception to the correspondence between eastern/western genetic clades and solitary/gregarious phase. The ideal way to determine both the degree of plasticity (i.e. the level of environmental change needed to induce a population to switch phase), and whether the thresholds for phase change differ between populations, would be to perform reciprocal transplant experiments between several eastern solitary and western gregarious field sites, with several replicates of different rearing densities. Such a study, perhaps even performed over several years, could distinguish between abiotic environmental factors (eg. climate) and biotic factors (eg. density) that influence phase change. For example, would solitary Mormon cricket eggs transplanted into a western environment at high density develop a gregarious phenotype, and vice versa? Differences in the ease of expression of the alternative phenotype may indicate asymmetry in the threshold for phase change. Such an experiment would also quickly indicate if the phenotypic differences between solitary and gregarious phases were in fact fixed. Alternatively, lab studies rearing Mormon crickets hatched from eggs collected in solitary versus gregarious populations under controlled density and environmental regimes could also be used to test the thresholds inducing phase switching. It would be particularly interesting to investigate any epigenetic effects on eggs laid during intense migratory activity in gregarious bands as opposed to eggs laid in low-density solitary populations.

Future directions

The geographic division between eastern and western clades of Mormon crickets corresponds almost exactly with one of Remington's (1968) purported North American suture zones, and this zone is one of only two that has withstood a more recent GIS analysis with added data by Swenson and Howard (2004). It is uncertain whether populations from the western clade ever encounter populations within the eastern clade; I have neither observed this during my field work nor have I read or heard any accounts of gregarious populations hybridizing with solitary populations on the eastern slope of the Rockies. Thus there may be little opportunity for contact between these geographically isolated clades, although a tantalizing clue as to the outcome of such contact can be gained from study of the Little Brush Creek (LBC) population in Utah. LBC is the only solitary population that clusters within the western clade, and during my first season of fieldwork, a gregarious band of Mormon crickets was observed passing through the solitary population. This raises an intriguing question: is the solitary LBC population genetically indistinguishable from the other—gregarious—western clade populations because it is simply expressing the solitary phase polyphenism, or does it have a separate origin and the mtDNA similarity is a result of continuous introgression from gregarious bands? As before, studies investigating the threshold for phase change in this species would help answer this question, however, an alternative tactic may be to assess the degree of reproductive isolation between solitary and gregarious phases.

Any investigation of population divergence or incipient speciation events must take into account the role (if any) of reproductive isolation in geographically isolated populations because geographic separation may only reduce gene flow as opposed to eliminating it outright (eg. Rice and Hostert 1993, Lee 2000). Extensive genetic divergence is not a prior requisite for the evolution of pre- and post-copulatory reproductive isolation (Howard 1999, Gleason and Ritchie 1998, Tregenza *et al.* 2000 and 2002, Dixon *et al.* 2003), and sexually selected traits like male courtship song are expected to diverge rapidly in allopatric populations (West-Eberhard 1983, Andersson 1994, Mandelson and Shaw 2005). Other studies suggest that long periods of isolation in allopatry are also associated with strong post-copulatory reproductive isolation (Tregenza 2002).

Both pre- and post-copulatory reproductive isolation should be assessed in Mormon crickets. Results that I have not included in this thesis because of concerns about methodological flaws (discussed further in the next section) suggest that certain combinations are more favoured in reciprocal mating experiments with Mormon crickets. When solitary and gregarious Mormon cricket males and females were paired in the four possible combinations, successful mating (i.e. spermatophore transfer) occurred most frequently in the solitary male/gregarious female combination. Gwynne's (1981, 1984, 1993) work established that role reversal occurred when the relative value of the male spermatophore was elevated due to high density and low nutrient availability, a situation common in gregarious populations. These results confirm the prediction that a role-reversed gregarious female paired with a solitary male would result in the least discriminating mating pair. This, along with observations at the solitary LBC site, suggests that mating between solitary and gregarious phases may occur readily, although sperm utilization patterns may favor offspring sired by same-population males if hybrid sterility or inviability has selected for post-copulatory reproductive isolation.

Darryl Gwynne and I have started a small pilot study on sperm competition in Mormon crickets (this work is uncompleted). We captured gregarious females that were seen to be carrying a spermatophore in the field to ensure that they were mated at least once. After several days, each female was paired with a gregarious (or in one case solitary) male that had been kept in captivity for several days with an excess of food, and they were allowed to mate. The intent of this study is to determine a baseline measurement of P_{last} (the proportion of offspring sired by the last male to mate) and to assess the extent of polyandry in the field. Does P_{last} decrease when females are mated with males from the other phase? A future goal of Mormon cricket studies should be to assess not only the potential for sperm competition, but also whether sperm of same-phase males is used preferentially over that of different-phase males.

This study should be constructed to test the hypothesis that the strength of reproductive isolation may differ between solitary and gregarious populations as a result of asymmetries in mating interests of solitary and gregarious individuals. In Mormon crickets, there is evidence that role-reversal in western-clade gregarious populations has been consistent due to both their high densities and habitat and environment differences, which makes this a good system to test whether these differences predict asymmetrical introgression between the eastern and western clades (Panhuis *et al.* 2001). Asymmetrical introgression has been observed in hybrid zones of

Chorthippus parallelus (Bella *et al.* 1992) and in a North American field cricket (Harrison 1986) but this was suggested to be an effect of asymmetrical homogamy as opposed to differential mating interests.

Finally, despite (or rather because of) the debate over whether pre- or post-copulatory reproductive isolation is more likely to evolve first in allopatric populations (Tregenza 2002), pre-copulatory reproductive isolation in Mormon crickets should be tested. Ritchie *et al.* (1997) found that song races in *Ephippiger ephippiger* corresponded to subspecific variation uncovered by RAPDs, and that females show a strong preference for songs of their own race (Ritchie 1991). Drift may have induced divergence in male song and female preferences during periods of allopatry, and hybrid inviability upon secondary contact can select for greater recognition of same-phase song to prevent hybrid matings. If this is the case, we predict that female Mormon crickets should show the pattern seen in other Tettigoniids (eg. Ritchie 1991) and prefer song of same-phase males. However, if hybrid inviability has not developed between solitary and gregarious Mormon crickets, or a lack of secondary contact has not reinforced song preferences in females, a different prediction may be made. Song can be an indicator of male quality as has been suggested in field crickets (Simmons 1995, Simmons and Ritchie 1996, Scheuber *et al.* 2003a) and in this case, we predict that both solitary and gregarious females would show the strongest preference for a "high quality" song regardless of phase (an example of such a song might be one that reflects the age of the male, eg Ritchie *et al.* 1995). Experiments to test these hypotheses are of course predicated on the fact that there are differences in song structure or frequency between solitary and gregarious males, but as chapter 5 and the following section demonstrate, it is unclear whether the differences uncovered are both consistent from year to year and whether they are significant enough to allow for female discrimination.

Pitfalls I have encountered

Not all of the projects I undertook during the course of this research worked out. Several were subject to methodological failures that I believe were fatal flaws, but it is important to note what these projects were and why they failed, as this contributed to my understanding of the hypotheses being tested. Additionally, the manuscripts that comprise this thesis were not immune to difficulty and limitations.

During the 2004 season of fieldwork, I designed and performed playback experiments in the field. We used one representative recording from a gregarious male and one from a solitary male. The main differences between the two songs that year were the chirp rate and peg strike rate, so it was unclear whether these differences would be discernable to phonotaxing females. We performed choice and no-choice experiments and in the former found that neither gregarious nor solitary females showed a preference for either song. However, the results of this experiment were most likely invalid because in the no-choice experiments, females did not show a tendency to phonotax towards male song, whereas other Decticines show a strong phonotactic response towards male song (eg. Ritchie 1991). This indicates that there was a problem with our experimental set up, which could have included: inadequate playback speakers, too much echo or stressed females. Additionally, in 2005 there was a difference in carrier frequency between solitary and gregarious males (reported in chapter 5), which indicates that song parameters may vary from year to year and the song samples in our choice experiments did not provide enough of a contrast for females to evaluate.

Future playback experiments should be performed in an anechoic chamber in the lab using synthetic male songs, and male song in the wild should ideally be evaluated on a yearly basis to determine the extent of long-term temporal variation in carrier frequency. It will be difficult to design experiments that specifically examine solitary versus gregarious song, because the song traits that vary between solitary and gregarious males may also convey information about male condition (eg. Scheuber *et al.* 2003a, 2003b). For example, it would be difficult to distinguish the selection pressures that have favoured the evolution of a female preference for lower frequency song if lower frequency is correlated with both gregarious males (eg. the preference has arisen as a result of selection for increased mate recognition) and males with larger body size (eg. the preference has arisen as a result of selection on females to choose high-quality males) or both. This difficulty could be surmounted by designing playback experiments with synthetic songs that vary in all combinations of parameters, and using a multivariate analysis to demonstrate whether females of a given phase prefer song traits that are most consistent with the male song of that phase. For example, would solitary females prefer higher frequency songs, songs with faster chirp rates, and songs with more peg strikes per chirp?

Another frustration we encountered was the rearing of Mormon crickets. Sperm competition experiments were confounded by the difficulty of keeping eggs healthy until

embryonic development or hatching. Eggs laid by stressed females may not even have been fertilized (Ritchie pers. comm.), and breaking diapause in Tettigoniid eggs is notoriously difficult (Vaheed pers. comm.). In the sperm competition pilot study presently underway, Greg Sword is using the previously successful method of burying eggs outdoors to approximate diapause conditions as closely as possible (Sword pers. comm.).

Finally, we had hoped to include a strong nDNA component of our genetic analyses, which rely mostly on mtDNA sequencing. We developed microsatellite markers from a library created by Leon Hockham (see chapter 3), but this process was arduous and resulted in a low number of useful markers and a high number of markers with null allele problems. This is not an uncommon problem for Orthopteran species (eg. Hockham *et al.* 1999, Zhang *et al.* 2003). We also initially tried sequencing a nuclear gene, ITS, but variability in this region was so low as to render it phylogenetically uninformative.

The microsatellite analysis based on a limited number of loci (chapter 4) was informative and corresponded well to the mtDNA patterns uncovered. I would be particularly interested in further examining sex-biased dispersal, as the pattern we uncovered in the gregarious clade indicates that males move further and interpopulation migrants were more likely to be sampled, but this analysis should be done with a greater number of microsatellite loci to ensure that the pattern is consistent and it should be done in solitary populations as well. The microsatellite loci developed will be of great use in assessing paternity for sperm competition experiments; however future phylogeographic investigations may benefit from the use of a whole-genome approach such as AFLPs (eg. Knowles and Richardson 2005).

APPENDIX: CAN SEXUAL SELECTION THEORY PREDICT HOMOSEXUAL MALE PARTNER CHOICE?

NW Bailey, ŁK Michalczyk

Abstract

Examples of male homosexual behaviour in animals are well-documented, but most studies have focused on proximate mechanisms to infer ultimate explanations of how such a seemingly disadvantageous trait can be evolutionarily maintained in a population. A more fruitful approach may be to study male-male mating dynamics to provide clues of possible ultimate explanations. This review asks: what governs the choices that males make when seeking a male partner? We present three hypotheses about how to classify male homosexual behaviour plus three predictions that can be made about homosexual male choice under each. Specifically, we posit that homosexual male behaviour can be either adaptive or non-adaptive, depending on the species in question, and adaptive explanations give rise to specific predictions about characteristics of the male mates that homosexual males prefer. We further investigate non-adaptive male homosexual behaviour and predict that homosexual male partner choice in such instances will follow one of two patterns, based on the idea that either a) homosexual behaviour results from a simple switch in orientation, i.e. the sex towards which males direct mating behaviour, or b) that homosexual behaviour results from a broader "feminization" of many traits involved in mating behaviour, including partner trait preferences. Recent literature on male choice in role-reversed species, female choice and sexual selection theory—plus research examining male homosexual behaviour in a range of animals—provide the foundation to support our three predictions, and we discuss future directions for empirical research based on these theoretical considerations.

Introduction

Studies of homosexual mating behaviour have been focused on explaining the causes of homosexual orientation (eg. LeVay 1991, Hamer *et al.* 1993, Hu *et al.* 1995), and the majority of this politically-charged research has been directed towards humans or the more easily studied *Drosophila melanogaster*. Many studies, most of which focus on humans, have investigated proximate mechanisms of homosexual orientation in individuals. Some of these are summarized in box 1. Inferring ultimate causation from proximate explanations can lead to confusion, however, and here we present an alternative approach. This review asks how sexual selection theory might inform the study of male-male mating dynamics in non-human species, and how that in turn may inform ultimate explanations of homosexual behaviour. The applicability of sexual selection theory has been demonstrated and debated for heterosexual matings, and it is a useful tool for predicting such factors as the dynamics of pre- and post-copulatory reproductive isolation, courtship roles, the extent of courtship displays—including ornamentation and courtship song—and female preferences for such displays. Recent publications have documented male-male mating behaviour or copulation attempts in numerous non-human species, including sheep, rats, guppies and other insects, but what governs the choices that males make when seeking a male partner?

It is important to note that "homosexual mating behaviour" and "homosexual orientation" are not necessarily the same thing. It is only possible to make this distinction in humans, because all we can observe in non-human species are matings and displays directed towards other males. Here we focus on homosexual mating behaviour, i.e. any behaviour directed from one male towards another which is typically directly involved in producing a heterosexual copulation, such as courtship displays or actual copulation attempts. This paper is thus not concerned with the direct proximate mechanisms underlying homosexual mating behaviour, i.e. the processes in the brain effecting the behaviour, but if we are to ask what regulates the choices males make when mating or attempting to mate with another male, it is necessary to consider his indirect reasons for doing so.

In heterosexual matings, the intention of mating obvious: it is to pass on as many of his genes via as many progeny as possible and to ensure their survival. This basic selective force has spawned innumerable mechanisms by which males maximize their mating potential,

BOX 1: A few hypothesized proximate mechanisms of homosexual orientation

Proximate and ultimate explanations for the existence of homosexual orientation are very often confused and although it is not necessarily the case, they can sometimes appear to be mutually exclusive. Here we describe several proximate mechanisms that can be found in the literature. It is worth remembering that proximate mechanisms can be complex and may occur on more than one level: direct or indirect. Since all behaviours originate in the brain, we can expect that some differences in brain structure and/or chemistry should be noted between hetero- and homosexual males. An example of that kind of **direct proximal** explanation is given in item 1. However, differences in brain structure/chemistry must have other causes. These other **indirect proximal** explanations may be different and some examples of them are given in items 2-5. Thus, direct and indirect proximate causes taken together give a full proximate explanation.

1) Differences in size of anterior hypothalamus, a brain structure influencing sexual behaviour, have been shown in homosexuals in studies of humans and sheep (respectively, LeVay *et al.* 1991, Roselli *et al.* 2004). This correlation is contentious, however, as such differences can be interpreted as causes or effects of homosexual orientation, and replication studies (eg. Byne *et al.* 2000) have disputed the initial findings of LeVay *et al.* (1991) and Swaab and Hofman (1990).

2) Genetic evidence from linkage studies in humans suggests an association between male homosexual orientation and a marker on the X chromosome (Hamer *et al.* 1993) although follow-up studies have failed to replicate these findings (Rice *et al.* 1999). Similarly, DuPree *et al.* (2004) found no association or linkage between the gene for *aromatase*, which is vital to sexual differentiation in the brain, and male homosexual orientation in humans. Different mutations in the *fruitless* gene of *Drosophila melanogaster* give rise to mutant flies displaying varying degrees of homosexual behaviour (eg. Yamamoto *et al.* 1996).

3) Fraternal birth order has been suggested to predict homosexual orientation in between 15% and 29% of human male homosexuals, and this may be a result of the aggregation of maternal antibodies during repeated pregnancies where the child is male, and the effects of these antibodies on sex differentiation in fetus's brain (eg. Blanchard 2004). Having more older brothers thus increases the chances of a male being homosexual (Blanchard 2004, Bogaert 2003, Camperio-Ciani *et al.* 2004).

4) Epigenetic effects have been suggested as a potential pathway to homosexual brain differentiation (Blanchard 2004), as their importance in the development of other adult behaviours has been demonstrated in non-human organisms such as mice (Francis *et al.* 2003)

5) Prenatal stress may have a small but significant effect on the sexual orientation of human offspring due to disruption in the brain sex-differentiating hormones during pregnancy (see Ellis and Cole-Harding 2001).

including complex mate guarding behaviours, sperm competition, direct and indirect paternal investment in offspring and intrasexual competition. Male-male copulations do not result in the direct passage of genes to the next generation, however, and researchers have attempted to reconcile the paradox of how such a seemingly disadvantageous trait can be evolutionarily maintained in a population, provided it is not simply a case of mistaken identity (Macías Garcia and Valero 2001). One school of thought is that male homosexual behaviour is adaptive, but another school of thought purports that homosexual behaviour is not adaptive and may be a peculiar by-product of evolution or development (for examples see Bobrow and Bailey 2001, Camperio-Ciani *et al.* 2004). By examining documented examples of male homosexuality in non-human animals and applying the lessons of sexual selection theory, we will generate three hypotheses about potential evolutionary origins of homosexual behaviour—representing indirect, rather than direct, proximate mechanistic explanations—which lead to three novel and testable predictions about what traits males prefer when they initiate homosexual matings.

Male homosexual matings may have different outcomes in different species. We can divide homosexual male mating behaviour, from the perspective of the male making the attempt (who we refer to as the focal male), into two broad categories: 1) mating behaviour which will incur a net direct or indirect benefit to the focal male and 2) mating behaviour which will incur a net cost to the focal male either through direct costs such as energy expenditure or indirect costs resulting from a decrease in reproductive success. The first category therefore assumes that homosexual behaviour is an evolved adaptation, whereas the second category assumes that homosexual behaviour—although it may be the product of evolution—has not evolved as an advantageous trait for the focal male (genes influencing homosexual orientation may however be adaptive in other individuals – e.g. females). Characteristics that homosexually oriented males prefer in other males will thus greatly depend on which category the mating behaviour falls into: males that are mating to incur a benefit to themselves may have different preferences than males that direct mating attempts towards other males even when such activity decreases focal males' reproductive success.

Adaptive male homosexual mating behaviour

Adaptive explanations for the presence of homosexual behaviour in males may be easiest to examine, as there are a number of examples in the literature of how male-male courtship behaviour can be either beneficial to the focal male or costly to the receiving male. In the desert locust *Schistocerca gregaria*, males release phenoacetylnitrile (PAN), a volatile pheromone developed upon sexual maturity, to conceal female pheromones that would otherwise attract competing males (Seidelmann and Ferenz 2002). In addition to acting as a mate guarding mechanism, the release of PAN may also avert homosexual mating attempts in crowded swarms by marking a male—in addition to his female mate—as "taken", thereby repelling other males and preventing homosexual encounters (Seidelmann and Ferenz 2002). This and other evidence from the housefly *Musca domestica* (Adams and Holt 1987) indicates that males in some species may have evolved sex discrimination and homosexual courtship inhibiting abilities because being on the receiving end of homosexual mating attempts is costly.

The converse of this is that in some cases homosexual mating behaviour may be beneficial for the focal male. In the termite species *Reticulitermes speratus*, tandem running occurs when male and female alates that have broken off their wings search for a suitable nesting site, but both male and female homosexual tandem running has been observed in this and other termite species. Matsuura *et al.* (2001) have suggested that, rather than homosexual mating behaviour being costly to the focal males, it may in fact protect individuals from predation because of selfish herd effects. Exclusively male or exclusively female tandems dilute the risk of predation while individuals in those tandems are searching for a member of the opposite sex. Another example comes from red-sided garter snakes, *Thamnophis sirtalis parietalis*. Young male garter snakes go through a period during which they mimic females by expressing female-typical skin lipids. Shine *et al.* (2000) propose a number of mechanisms by which male garter snakes that express female skin lipids and passively court other males may benefit from such interactions, and these include increasing the energy expenditure of rival males and distracting them from potential female mates. What is noteworthy in garter snakes is the fact that after a period during which males exclusively court other males, they then switch to courting females when conditions are right. The adaptive advantages of homosexual behaviour in these examples are varied: to name a few, homosexual mating attempts may a) distract rivals, thereby increasing

the focal male's chances of mating with an unguarded or unmated female b) impose costs on rivals by engaging them in energetically wasteful homosexual mating activity or c) protect against predation.

If in such systems male-male courtship provides an adaptive advantage in intrasexual competition, we would expect males that engage in homosexual mating behaviour to enjoy increased reproductive success. Logically at some point in their life they will engage in heterosexual mating encounters. If homosexual male behaviour is adaptive, males cannot remain exclusively homosexual unless the inclusive fitness benefits they gain exceed the cost of not mating. However, this seems unlikely if there is a clear advantage in intrasexual competition, for example, the distraction of rivals, and males mate with females later on in life as with garter snakes or locusts (Shine *et al.* 2000, Seidelmann and Ferenz 2002), Also, males should economise their homosexual encounters to minimize the potential costs of the encounter to themselves and maximize the potential costs of the encounter to rival males. The evolution of sex discrimination factors and male countermeasures to homosexual courtship, as in desert locusts (Seidelmann and Ferenz 2002), suggests that these costs are not insignificant. As such, homosexual male partner choice in these cases should follow the 'rules of engagement' for general male-male aggression: males will be expected to attempt mating with other males when the benefits for the focal male are likely to exceed the costs. Costs for the focal male in homosexual mating attempts will be equivalent to the costs of intrasexual aggression, namely energy expenditure, potential harm or fatality and distraction from mating with females. As Shine *et al.* (2000) demonstrated, male garter snakes minimize the costs and maximize the benefits by engaging in homosexual mating behaviour just after emerging from hibernation, at a time when their locomotor abilities are not sufficient for mating with females.

Non-adaptive homosexual male mating behaviour

In some cases, however, homosexual behaviour does not so clearly serve an adaptive purpose. Homosexual behaviour in rams is well-documented (eg. Price *et al.* 1988) but seems not to incur a direct benefit to males as has been suggested above with locusts, termites and garter snakes. Rather, rams that are male-oriented display typical male courtship and copulatory behaviours and that behaviour is unlikely to be related to hormone levels in adults (Roselli *et al.*

2004) but may reflect underlying neural differences (Perkins *et al.* 1995). Male-oriented rams are more or less exclusively male-oriented, and this behaviour does not change in response to external population or environmental factors (Zenchak *et al.* 1981). In cases such as this, there is no clear outcome of homosexual mating except that homosexual males will invariably have lower than average reproductive success.

Predictions made about preferences depend on what assumptions are made about the indirect proximate mechanism of homosexual behaviour in a population or species—be it genetic, social, environmental or some combination: 1) if mating preferences in homosexually oriented males have not diverged to the extent that their behaviour has diverged, that is, if the only thing that is different about homosexual males is the sex to which they direct their courtship and mating attempts, then we might expect them to retain the mating preferences of heterosexual males. In such cases homosexual males would prefer other males that reflect the qualities heterosexual males prefer in female mates, if indeed male preference even exists. 2) If homosexual behaviour in males is a result not only of a simple switch in sexual preference but also represents changes in a broader suite of traits that are inherited (or learned, or environmentally induced) together, i.e. homosexual behaviour is correlated with a "feminization" of many behavioural characteristics, males may prefer the same characteristics in a mate that females do. Currently there is not enough research to definitively conclude that the existence of homosexual behaviour is more consistent with one of these patterns than the other, and it bears consideration that these two competing hypotheses about homosexual male choice may not be mutually exclusive and could be species-dependent. Nevertheless, each hypothesis generates predictions that would be easy to test.

Predictions about non-adaptive homosexual male partner preference

What studies can we examine to refine the predictions with respect to the first non-adaptive hypothesis, which predicts that homosexual male preferences will mirror heterosexual male preferences (if they exist)? Typically females are choosier than males because of their higher parental investment (Queller 1997). One way to gain insight about heterosexual male mating preferences is to examine species in which sex roles are reversed. The theory of sexual selection posits that the sex with the lower potential reproductive rate will be the 'choosy' sex,

and this sex is usually the females due to greater maternal investment in offspring (Trivers 1972, Queller 1997). This has been confirmed through experimental manipulations of parental investment in spermatophore-producing insects (Gwynne and Simmons 1990), in which the potential reproductive rate of males was manipulated by altering food availability in test populations of a katydid species. Low food availability caused fewer males to produce spermatophores, thereby increasing males' relative parental investment (Gwynne and Simmons 1990). Evidence from this, and another role-reversed katydid species, indicates that males prefer heavier females because body weight correlates with ovary weight and number of mature eggs, and thus heavier females are likely to be more fecund (Gwynne 1981). Male preference for heavier females has been demonstrated in other species, including assassin bugs (Thomas and Manica 2005) and garter snakes (Lemaster and Mason 2002), and males in species such as the two-spotted gobi (*Gobiusculus flavescens*) and dance flies (*Empis borealis*) may also respond to female ornaments that honestly signal female fecundity (Amundsen and Forsgren 2001, LeBas *et al.* 2003).

The evidence from role-reversed species indicates that when males are the choosy sex, they are likely to chose females based on their perceived fecundity, and although a commonly used measure of female fecundity is weight, other honest signals may evolve in role-reversed systems. These preferences, however, are likely to be species-specific, and it bears mentioning that males have also been shown to prefer virgin or younger females, more receptive females, or unfamiliar females. If the mate characteristics that homosexual males prefer have not become decoupled from those that heterosexual males prefer (i.e. the only difference is which sex they direct mating attempts towards), then the first hypothesis predicts that homosexual males will prefer to mate with males showing traits that most closely resemble the female traits that role-reversed males prefer, and generally they prefer larger, heavier, and therefore more fecund females, or females with exaggerated sexually selected ornamentation signaling their fecundity.

According to our second non-adaptive hypothesis, however, mate characteristic preference could be closely tied to homosexual mating behaviour, with the two being inherited (or learned, or environmentally induced) together in a more "feminised" male, rather than becoming uncoupled as in the first hypothesis. If in this case mating preferences of homosexual males more closely resemble the mating preferences of females, then a different prediction arises. The potential for males under strong sexual selection to evolve ornamentation or

courtship displays is great, and instead of gauging apparent fecundity, choosy females can evaluate male fitness or quality based on his ornamentation, which may honestly signal his quality. Homosexual males whose mating preferences are homologous to female mating preferences will therefore be predicted to choose other males based on their ornamentation, courtship displays, songs or whatever characteristic may be under sexual selection. The partner preferences of homosexual males will thus mirror those of females, and they will prefer males that are more elaborately ornamented, more competitive or fitter, however that may be reflected in the species in question.

Interestingly, if male homosexual behaviour is common in a species (as it is in sheep: it is estimated that roughly 9% of rams are male-oriented (Price *et al.* 1988)) and male engaging in such behaviour prefer the same characteristics in male mates that females prefer, at least a small degree of competition might be expected between homosexually-pairing males and heterosexual females. If male quality varies, the removal of these males from the pool of potential mates for females—and their subsequent distraction and monopolisation of good-quality males—would skew the operational sex ratio. Competition would then be expected between homosexually-pairing males and heterosexual females because they both prefer the same high-quality males, who are now a limited resource. If competition is strong enough, this could even lead to sub-optimal mating by females.

Empirically testing homosexual male partner preference hypotheses

Here we have presented three hypotheses about how to classify male homosexual behaviour and the predictions that can be made about male choice under each of them. **Adaptive competition hypothesis:** Males displaying homosexual mating behaviour in species where homosexual matings are an adaptive, competitive mechanism will be predicted to discriminate amongst males for copulation attempts in a pattern that reflects the choices males typically make when displaying other forms of aggression such as fighting or competitive displays. Furthermore, these males will mate with females at some point in their life. What distinguishes the prediction made by this hypothesis from the following two is that homosexual male partner preferences will be determined relative to the fitness of the male doing the choosing. To optimize his benefits, a male engaging in intrasexual competition must economize his aggression

towards other males based on his own competitive abilities, and the same applies if homosexual mating behaviour is simply a form of intrasexual competition. For example, a small focal male may choose to direct mating behaviour towards the smaller of two male subjects so that he minimizes the potential costs, i.e. risk of injury, but a larger focal male may prefer the larger subject because the risk to him is less.

In some species homosexual male encounters may bring no apparent benefit to males displaying that behaviour, however, and may indeed decrease their reproductive success. There are two hypotheses within this category about how male preferences may operate. **Non-adaptive, mirror heterosexual male preferences hypothesis:** If homosexual mating behaviour is merely a matter of switching the focus of male mating behaviour from females to males, males engaging in such behaviour will be predicted to retain preferences (if any) that heterosexual males exhibit, namely traits that would normally reflect fecundity and quality in females. In contrast to the first hypothesis, if heterosexual males prefer large fecund females in a given species, homosexual focal males will always prefer larger males, regardless of their own size. **Non-adaptive, mirror female preferences hypothesis:** If male homosexual mating behaviour is not simply a switch in focus from females to males but reflects a change in a number of broader characteristics such as the processes governing which traits are evaluated in a potential mate and the optimal trait values, males engaging in homosexual behaviour would be predicted to display more female-like preferences when choosing males to direct mating behaviour towards. Again, in contrast to the first hypothesis, homosexual male partner preference will be independent of the focal male's own fitness.

Theoretical predictions are interesting, but are more useful if they can be applied to empirical systems. A recent study of guppies, *Poecilia reticulata*, suggests that the social environment of male guppies can induce homosexual behaviour if males are kept in all-male cohorts (Field and Waite 2005). Males kept in such conditions for 15 weeks spent significantly more time performing courtship displays towards males than females when given a choice between the two. Additionally, after reintroduction to a mixed sex cohort, males still retained a significant amount of homosexual preference. The authors claim that the male behaviour they were using to assess male preference was solely courtship behaviour, which is used typically in sexual displays as opposed to aggressive manoeuvers. Regardless of the fact that they observed male sexual displays directed towards other males as opposed to females, they cannot determine

the intent of those displays. It is possible that the focal males derived a benefit, or to put it another way, imposed a cost, upon the males to whom they displayed, and that the development of these behaviours was influenced by their social environment. For example, males reared in all-male environments may perceive there to be more male-male competition for mating simply because of the highly skewed sex ratio, and as a result, they may have adjusted their behaviour to include more intrasexual competitive displays. This explanation is only feasible, however, if males that display more towards other males would experience greater reproductive success as a result. The costs of such displays could be considerable if the focal male expends too much energy and is distracted from finding females to mate with. The study implied that there was significant retention of homosexual behaviour by males in same-sex cohorts, even after exposure to females, but its scope was too narrow to determine whether the development of homosexual behaviour affected their future reproductive success and what the costs and benefits of such behaviour might have been.

One way to obtain more information about the dynamics of homosexual behaviour in a population or species like these guppies would be to explicitly test the three predictions about male choice outlined above. That is, if males who exhibit homosexual behaviour either as a result of their social environment or some intrinsic biological predilection were given a choice not between a male and a female, but between males varying in quality, the pattern shown by focal males, in addition to measurements of focal male fitness, could inform the indirect proximate explanation underlying homosexual matings. Such experiments necessitate a study species in which male quality varies, and is either honestly or dishonestly reflected by ornaments, courtship displays or song. Guppies, or any other organism in which sexual selection has driven the evolution of male ornaments, are well-suited to this. If male guppies who show a consistent preference for males over females were given a choice between two males with varying degrees of ornamentation, for example, the overall pattern of homosexual male choice may indicate which of the three hypotheses is more valid. It must be noted that individual males within a population may perform homosexual courtship behaviour or matings for different reasons, in which case such a test could probably not distinguish between them. If male guppies showing a consistent preference for males over females displayed patterns of partner preference more in line with patterns of aggression, and their choices varied with their own fitness, then we may tentatively conclude that homosexual behaviour in those individuals is a result of

intrasexual competition. If, however, focal males preferred males in a pattern more concordant with role-reversed heterosexual male preference or female preference, and their choices are independent of their own fitness, it would agree with Field and Waite's (2005) assertion that male homosexual behaviour in *P. reticulata* is not antagonistic and therefore is not indicative of intrasexual competition.

Conclusions

Studies of sexual selection theory have largely ignored homosexual dynamics, probably because it is difficult to distinguish different modes of homosexual behaviour in different species, and because research in these areas has been largely directed towards humans. What we propose, however, is that we can go some way towards surmounting these difficulties by taking an alternative approach. Homosexual mating behaviour—or the tendency to learn such behaviour, depending on whether it is genetically, socially, or environmentally induced—may have evolved as an adaptive mechanism of intrasexual competition. However, in some cases its costs outweigh its benefits and an adaptive explanation is not plausible. We can therefore learn about the dynamics of male homosexuals in populations or species not by studying the direct proximate mechanisms that induce their behaviour, but rather by studying the partner choices they make. Here we have proposed several hypotheses about homosexual male choosiness, based on observations in species in which male homosexual behaviour has been studied, and also in role reversed species where males are the choosy sex. These hypotheses may not be mutually exclusive, which bears consideration, but provide us with a starting point to examine homosexual mating dynamics by generating easily tested predictions.

Acknowledgements

The authors would like to thank Constantino Macías Garcia and Michael G. Ritchie for valuable comments on the manuscript, and we are also grateful to William V. Bailey for his feedback during the early stages of writing.

REFERENCES

Adams TS, Holt GG (1987) Effect of pheromone components when applied to different models on male sexual behaviour in the housefly, *Musca domestica. Journal of Insect Physiology* **33**:9-11.

Amundsen T, Forsgren E (2001) Male mate choice selects for female coloration in a fish. *Proceedings of the National Academy of Sciences of the USA* **98:**13155-13160.

Andersson M (1994) Sexual selection. Princeton, New Jersey: Princeton University Press.

Anonymous (1991) Mormon cricket: *Anabrus simplex* Haldeman. Wyoming Agricultural Experiment Station Bulletin 912.

Associated Press (2003) Nevada county declares cricket emergency. June 6, 2003.

Avise JC, Arnold J, Martin Ball R, Bermingham E, Lamb T, Neigel JE, Reeb CA, Saunders NC (1987) Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. *Annual Review of Ecology and Systematics* **18**:489-522.

Avise JC, Ball RJM (1990) Principles of genealogical concordance in species concepts and biological taxonomy. In: Oxford Surveys in Evolutionary Biology (ed. Futuyma D and Antonovics J). Oxford University Press, Oxford, UK.

Avise JC, Walker D (1998) Pleistocene phylogeographic effects on avian populations and the speciation process. *Proceedings of the Royal Society of London Series* B **265**:457-463.

Avise JC (2000) Phylogeography. Harvard Univ. Press, Cambridge, MA.

Ayoub NA, Riechert SE (2004) Molecular evidence for Pleistocene glacial cycles driving diversification of a North American desert spider, *Agelenopsis aperta. Molecular Ecology* **13**:3453-3465.

Bailey NW, Gwynne DT, Ritchie MG (2005a) Are solitary and gregarious Mormon crickets (*Anabrus simplex*, Orthoptera, Tettigoniidae) genetically distinct? *Heredity* **95**:166-173.

Bailey NW, Hockham LR, Graves JA, Ritchie MG (2005b) Development and characterization of microsatellite loci in Mormon crickets (*Anabrus simplex*, Orthoptera, Tettigoniidae). *Molecular Ecology Notes* **5**:613-615.

Bandelt H-J, Forster P, Röhl A (1999) Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution* **16**:37-48

Barrowclough GF, Groth JG, Mertz LA, Gutiérrez RJ (2004) Phylogeographic structure, gene flow and species status in blue grouse (*Dendragapus obscurus*). *Molecular Eoclogy* **13**:1911-1922.

Beaumont MA (1999) Detecting population expansion and decline using microsatellites. *Genetics* **153:**2013-2029.

Beerli P (2002) MIGRATE: documentation and program, part of LAMARC. Version 1.7.1. Revised August 23, 2002. Distributed over the Internet, http://evolution.genetics.washington.edu/lamarc.html

Bella JL, Butlin RK, Ferris C, Hewitt GM (1992) Asymmetrical homogamy and unequal sex ratio from reciprocal mating-order crosses between *Chorthippus parallelus* subspecies. *Heredity* **68**:345-352.

Belwood JJ, Morris GK (1987) Bat predation and its influence on calling behaviour in neotropical katydids. *Science* **238**:64-68.

Bermingham E, Avise JC (1986) Molecular zoogeography of freshwater fishes in the southeastern United States. *Genetics* **113**:939-965.

Blanchard, R (2004) Quantitative and theoretical analyses of the relation between older brothers and homosexuality in men. *Journal of Theoretical Biology* **230**:173-187.

Bobrow D, Bailey JM (2001) Is male homosexuality maintained via kin selection? *Evolution and Human Behaviour* **22**:361-368.

Bogaert AF (2003) The interaction of fraternal birth order and body size in male sexual orientation. *Behavioral Neuroscience* **117**:381-384.

Bossart JL, Prowell DP (1998) Genetic estimates of population structure and gene flow: limitations, lessons and new directions. *Trends in Ecology and Evolution* **13**:202-206.

Boto L, Doadrio I (2003) Polymorphic microsatellites in two different species of the genus *Zoogoneticus* Meek, 1902 (Goodeidae, Actynopterygii). *Molecular Ecology Notes* **3**:70-72.

Britten HB (1996) Meta-analyses of the association between multilocus heterozygosity and fitness. *Evolution* **50**:2158-2164.

Brown C (2004) Pest detection and management programs: Grasshopper/Mormon cricket – background. http://www.aphis.usda.gov/ppq/ispm/grasshopper/background.html

Brown WM, George M, Wilson AC (1979) Rapid evolution of animal mitochondrial DNA. *Proceedings of the National Academy of Sciences of the USA* **76**:1967-1971.

Brunsfeld SJ, Sullivan J, Soltis DE, Soltis PS (2001) Comparative phylogeography of northwestern North America: a synthesis. In *Integrating ecology and evolution in a spatial context.* BES symposium vol. (ed. J. Silvertown & J. Antonovics), pp. 319-339. Oxford: Blackwells.

Butlin RK, Ritchie MG (1994) Behavior and speciation. Pp. 43–79 *in* P. J. B. Slater and T. J. Halliday, eds. Behavior and evolution. Cambridge Univ. Press, Cambridge, U.K.

Butlin RK, Tregenza T (1998) Levels of genetic polymorphism: marker loci versus quantitative traits. *Philosophical Transactions of the Royal Society of London Biology* **353**:187-198.

Byne W, Lasco MS, Kemether E, Shinwari A, Edgar MA, Morgello S, Jones LB, Tobet S (2000) The interstitial nuclei of the human anterior hypothalamus: an investigation of sexual variation in volume and cell size, number and density. *Brain Research* **856:**254-258.

Camperio-Ciani A, Corna F, Capiluppi C (2004) Evidence for maternally inherited factors favouring male homosexuality and female fecundity. *Proceedings of the Royal Society of London Series* B **271**:2217-2221.

Castellano S, Balletto E (2002) Is the partial Mantel test inadequate? *Evolution* **56**:1871-1873.

Chapco W, Kelln RA, McFadyen DA (1992) Intraspecific mitochondrial DNA variation in the migratory grasshopper, *Melanoplus sanguinipes. Heredity* **69**:547-557.

——— (1994) Mitochondrial DNA variation in North American melanopline grasshoppers. *Heredity* **72**:1-9.

Coltman DW (2005) Differentiation by dispersal. *Nature* **433**:23-24.

Cowan FT (1933) Mormon cricket control in Colorado. *Circular 57, office of state entomologist* (Colorado Agricultural College, Fort Collins, CO)

——— (1990) The Mormon cricket story. Montana State Univ., Agric. Exp. Station Special Report #31:7-42.

Cowan FT, Campbell SCM (1929) The Mormon cricket and its control. *Circular 53, office of state entomologist* (Colorado Agricultural College, Fort Collins, CO)

Cowan FT, Shipman HJ, Wakeland C (1943) Mormon crickets and their control. *US Department of Agriculture* Farmer's Bulletin No. 1928.

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

Crnokrak P, Roff DA (1998) Inbreeding depression in the wild. *Heredity* **83**:260-270.

DeFoliart GR, Finke MD, Sunde ML (1982) Potential value of the Mormon cricket (Orthoptera: Tettigoniidae) harvested as a high-protein feed for poultry. *Journal of Economic Entomology* **75**:848-852.

De la Vega-Salazar MY, Avila-Luna E, Macías-Garcia C (2003a) Ecological evaluation of local extinction: the case of two genera of endemic mexican fish, *Zoogoneticus* and *Skiffia*. *Biodiversity and Conservation* **12**:2043-2056.

De la Vega-Salazar MY, Avila-Luna EG, Macías-Garcia C (2003b) Threatened fishes of the word: *Zoogoneticus tequila* (Webb and Miller 1998) (Goodeidae). *Environmental Biology of Fishes* **68**:14.

DeSalle R (2005) Conservation genetics - Genetics at the brink of extinction. *Heredity* **94:**386-387.

DeWoody JA, Avise JC (2000) Microsatellite variation in marine, freshwater and anadromous fishes compared with other animals. *Journal of Fish Biology* **56**:461-473.

Dingle H (1985) Migration. In: Insect physiology, biochemistry and pharmacology. Vol. 9 behaviour (ed. Kerkut GA & Gilbert CI). Pergamon press, Oxford, UK.

Dixon SM, Coyne JA, Noor MAF (2003) The evolution of conspecific sperm precedence in Drosophila. *Molecular Ecology* **12**:1179-1184.

Dunkle R (2001) Testimony before the subcommittee on national parks, recreation, and public lands, committee of natural resources, United States house of representatives. http://resourcescommittee.house.gov/archives/107cong/parks/2001july19/dunkle.htm

DuPree MG, Mustanski BS, Bocklandt S, Nievergelt C, Hamer DH (2004) A candidate gene study of CYP19 (aromatase) and male sexual orientation. *Behaviour Genetics* **34**:243-250.

Elliot CJH, Koch UT (1985) The clockwork crickets. *Naturwissenschaften* **72**:150-153.

Ellis L, Cole-Harding S (2001) The effects of prenatal stress, and of prenatal alcohol and nicotine exposure, on human sexual orientation. *Physiology and Behaviour* **74**:213-226.

Endler JA (1977) *Geographic Variation, Speciation, and Clines.* Princeton, NJ: Princeton University Press.

Evans JD, Wheeler DE (2001) Gene expression and the evolution of insect polyphenisms. *BioEssays* **23**:62-68.

Ewing AW (1989) *Arthropod bioacoustics; neurobiology and behavior.* Ithaca, NY: Cornell University Press.

Excoffier L (2004) Patterns of DNA sequence diversity and genetic structure after a range expansion: lessons from the infinite-island model. *Molecular Ecology* **13**:853-864.

Felsenstein J (2004) *DNADIST v. 3.6. A program to compute distance matrix from nucleotide sequences.*

Field KL, Waite TA (2004) Absence of female conspecifics induces homosexual behaviour in male guppies. *Animal Behaviour* **68**:1381-1389.

Francis DD, Szegda K, Campbell G, Martin WD, Insel TR (2003) Epigenetic sources of ehavioral differences in mice. *Nature Neuroscience* **6:**445-446.

Freeman S, Herron JC (2001) Evolutionary analysis. Prentice Hall, Upper Saddle River, NJ

Fricke C, Arnqvist G (2004) Conspecific sperm precedence in flour beetles. *Animal Behaviour* **67**:729-732.

Fu Y-X (1997) Statistical tests of neutrality of mutations against population growth, hitchiking and background selection. *Genetics* **147**:915-925.

Geffen E, Anderson MJ, Wayne RK (2004) Climate and habitat barriers to dispersal in the highly mobile grey wolf. *Molecular Ecology* **13**:2481-2490.

Gillette CP (1904) Copulation and ovulation in *Anabrus simplex* Hald. *Entomology News* **15**:321-325.

Gleason JM, Ritchie MG (1998) Evolution of courtship song and reproductive isolation in the *Drosophila willistoni* species complex: do sexual signals diverge the most quickly? *Evolution* **52**:1493-1500.

Goudet J (2001) FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3). Available from http://www.unil.ch/izea/softwares/fstat.html.

Grant MW, Miles MA (2002) An insect molecular clock dates the origin of the insects and accords with palaeontological and biogeographic landmarks. *Molecular Biology and Evolution* **19**:748-61.

Greenfield MD (1990) Evolution of acoustic communication in the genus *Neoconocephalus*: discontinuous songs, synchrony and interspecific interactions. In *The Tettigoniidae: biology, systematics and evolution*. (Ed. WJ Bailey and DCF Rentz) Pp. 71-97. Heidelberg: Springer

Groombridge JJ, Jones CG, Bruford MW, Nichols RA (2000) 'Ghost' alleles of the Mauritius kestrel. *Nature* **403**:616.

Gurney AB (1939) Aids to the identification of the Mormon and Coulee crickets and their allies (Orthoptera: Tettigoniidae, Gryllacrididae). United States Department of Agriculture Bureau of Entomology and Plant Quarantine. June.

Gwynne DT (1981) Sexual difference theory: Mormon crickets show reversal in mate choice. *Science* **213**:779-780.

——— (1984) Sexual selection and sexual differences in Mormon crickets (Orthoptera: Tettigoniidae, *Anabrus simplex*). *Evolution* **38**: 1011-1022.

——— (1985) Role reversal in katydids: Habitat influences reproductive behaviour (Orthoptera: Tettigoniidae, *Metaballus* species). *Behavioural Ecology and Sociobiology* **16**:355-361.

——— (1993) Food quality controls sexual selection in Mormon crickets by altering male mating investment. *Ecology* **74**:1406-1413.

——— (2001) *Katydids and bush-crickets*. Comstock Publishing Associates: Ithaca, NY.

Gwynne DT, Bailey WJ, Annells A (1998) The sex in short supply for matings varies over small spatial scales in a katydid (*Kawanaphila nartee*, Orthoptera: Tettigoniidae). *Behavioural Ecology and Sociobiology* **42**:157-162.

Hamer D, Hu S, Magnuson VA, Hu N, Pattatucci AML (1993) A linkage between DNA markers on the X chromosome and male sexual orientation. *Science* **261:**321-327.

Hamill RM, Webb SA, Macías Garcia C, *et al* (Submitted) Comparison of genetic diversity at microsatellite loci in near-extinct and non-endangered species of Mexican goodeine fishes and prediction of cross-amplification within the family. (*Journal of Fish Biology*).

Hammond RL, Saccheri IJ, Ciofi C *et al.* (1998) Isolation of microsatellite markers in animals. In: *Molecular Tools for Screening Biodiversity* (eds Karp A, Ingram PG, Chapman DS), pp. 279-285. Chapman Hall, London.

Harpending HC (1994) Signature of ancient population growth in a low-resolution mitochondrial DNA mismatch distribution. *Human Biology* **66**:591.

Harrison RG (1986) Pattern and process in a narrow hybrid zone. *Heredity* **56**:337-349.

Henry CS (1994) Singing and cryptic speciation in insects. *Trends in Ecology and Evolution* **9**:388-392.

Hewitt GM (1996) Some genetic consequences of ice ages, and their role in divergence and speciation. *Biological Journal of the Linnean Society* **58**:247-276.

——— (1999) Post-glacial re-colonization of European biota. *Biological Journal of the Linnean Society* **68**:87-112.

——— (2000) The genetic legacy of the Quaternary ice ages. *Nature* **405**:907-913.

——— (2001) Speciation, hybrid zones and phylogeography – or seeing genes in space and time. *Molecular Ecology* **10**:537-549.

——— (2004) Genetic consequences of climatic oscillations in the Quaternary. *Philosophical Transactions of the Royal Society of London* B **359**:183-195.

Hey J, Machado CA (2003) The study of structured populations—new hope for a difficult and divided science. *Nature* **4**:535-543.

Hockham LR, Graves JA, Ritchie MG (1999) Isolation and characterization of microsatellite loci in the bushcricket *Ephippiger ephippiger* (Orthoptera: Tettigoniidae). *Molecular Ecology* **8**:905-906.

——— (2001) Variable maternal control of facultative egg diapause in the bushcricket *Ephippiger ephippiger*. *Ecological Entomology* **26**:143-147.

——— (2004) Sperm competition and the level of polyandry in a bushcricket with large nuptial gifts. *Behavioral Ecology and Sociobiology* **57**:149-154.

Hoelzel AR, Halley J, O'Brien SJ, *et al* (1993) Elephant seal genetic variation and the use of simulation models to investigate historical population bottlenecks. *Journal of Heredity* **84**:443-449.

Hoffman EA, MS Blouin MS (2004) Evolutionary history of the northern leopard frog: reconstruction of phylogeny, phylogeography, and historical changes in population demography from mitochondrial DNA. *Evolution* **58**:145-159.

Howard DJ (1993) Small populations, inbreeding and speciation. In: The Natural History of Inbreeding and Outbreeding: Theoretical and Empirical Perspectives (ed. Thornhill NW), pp. 118-142. University of Chicago Press, Chicago.

——— (1999) Conspecific sperm and pollen precedence and speciation. *Annual Review of Ecology, Evolution and Systematics* **30**:109-132.

Hu S, Pattatucci AML, Patterson C, Li L, Fulker DW, Cherny SS, Kruglyak L, Hamer D (1995) Linkage between sexual orientation and chromosome Xq28 in males but not in females. *Nature Genetics* **11**:248-256.

Hull JM, Girman DJ (2005) Effects of Holocene climate change on the historical demography of migrating sharp-shinned hawks (*Accipiter striatus velox*) in North America. *Molecular Ecology* **14**:159-170.

Ibrahim KM, Nichols RA, Hewitt GM (1996) Spatial patterns of genetic variation generated by different forms of dispersal during range expansion. *Heredity* **77**:282-291.

IUCN (2000) 2000 IUCN red list of threatened species. Gland, Switzerland and Cambridge, UK: IUCN

Kelley J (2002) Behaviour and conservation of endangered fish species. PhD thesis, University of St. Andrews.

Kephart SR (2004) Inbreeding and reintroduction: Progeny success in rare *Silene* populations of varied density. *Conservation Genetics* **5**:49-61.

Kidd DM, Ritchie MG (2001) A geographical information science approach to exploring variation in the bushcricket *Ephippiger ephippiger*. *GIS and remote sensing in Biogeography and Ecology, ed. Millington AC, Walsh SJ, Osborne PE, Kluwer Academic Publishers* **10(3)**:603-611.

Klicka J, Zink RM (1997) The importance of recent ice ages in speciation: a failed paradigm. *Science* **277**:1666-1669.

Knowles LL (2000) Tests of Pleistocene speciation in montane grasshoppers (genus *Melanoplus*) from the sky islands of western North America. *Evolution* **54**:1337-1348.

——— (2001) Did the Pleistocene glaciations promote divergence? Tests of explicit refugial models in montane grasshoppers. *Molecular Ecology* **10**:691-701.

——— (2004) The burgeoning field of statistical phylogeography. *The Journal of Evolutionary Biology* **17**:1-10.

Knowles LL, Maddison WP (2002) Statistical phylogeography. *Molecular Ecology* **11**:2623-2635.

Knowles LL, Richards CL (2005) Importance of genetic drift during Pleistocene divergence as revealed by analyses of genomic variation. *Molecular Ecology* **14**:4023-4032.

Koch UT, Elliot CJH, Schaffner K-H, Kleindienst H-U (1988) The mechanics of stridulation of the cricket *Gryllus campestris*. *The Journal of Comparative Physiology* A **162**:213-223.

Krakauer J (2003) Under the banner of heaven: a story of violent faith. Doubleday (New York)

Kumar S, Tamura K, Jakobsen IB, Nei M (2001) *MEGA2: Molecular Evolutionary Genetics Analysis*. Arizona State University, Tempe, Arizona, USA.

Lamb T, Jones TR, Wettstein PJ (1997) Evolutionary genetics and phylogeography of tassel-eared squirrels (*Sciurus aberti*). *Journal of Mammalogy* **78**:117-133.

Lande R (1988) Genetics and demography in biological conservation. *Science* **241**:1455-1460.

Lange CE, MacVean CM, Henry JE, Streett DA (1995) *Heterovesicula cowani* N. G., N. Sp. (Heterovesiculidae N. Fam.), a microsporidian parasite of Mormon crickets, *Anabrus simplex* Haldeman, 1852 (Orthoptera, Tettigoniidae). *Journal of Eukaryotic Microbiology* **42**:552-558.

Lansman RA, Avise JC, Aquadro CF, Shapira JF, Daniel SW (1983) Extensive genetic variation in mitochondrial DNAs among geographic populations of the deer mouse, *Peromyscus maniculatus*. *Evolution* **37**:1-16.

LeBas NR, Hockham LR, Ritchie MG (2003) Nonlinear and correlational sexual selection on 'honest' female ornamentation. *Proceedings of the Royal Society of London Series* B **270**:2159-2165.

Leberg PL (1993) Strategies for population reintroduction: effects of genetic variability on population growth and size. *Conservation Biology* **7**:194-199.

Lee, CE (2000) Global phylogeography of a cryptic copepod species complex and reproductive isolation between genetically proximate "populations". *Evolution* **54**:2014-2027.

Lemaster MP, Mason RT (2002) Variation in a female sexual attractiveness pheromone controls male mate choice in garter snakes. *Journal of Chemical Ecology* **28**:1269-1285.

LeVay S (1991) A difference in hypothalamic structure between heterosexual and homosexual men. *Science* **253**:1034-1037.

Lorch PD, Gwynne DT (2000) Radio-telemetric evidence of migration in the gregarious but not the solitary morph of the Mormon cricket (*Anabrus simplex* : Orthoptera : Tettigoniidae). *Naturwissenschaften* **87**:370-372.

Lorch PD, Sword GA, Gwynne DT, Anderson GL (2005) Radiotelemetry reveals differences in individual movement patterns between outbreak and non-outbreak Mormon cricket populations. *Ecological Entomology* **30**:548-555.

Lowe A, Harris S, Ashton P (2004) Ecological genetics: design, analysis, and application. Blackwell Publishing, Malden, MA

Lunt DH, Ibrahim KM, Hewitt GM (1998) MtDNA phylogeography and postglacial patterns of subdivision in the meadow grasshopper *Chorthippus parallelus*. *Heredity* **80**:633-641.

Lynch M, O'Hely M (2001) Captive breeding and the genetic fitness of natural populations. *Conservation Genetics* **2**:363-378.

Macías Garcia T, Valero A (2001) Context-dependent sexual mimicry in the viviparous fish *Girardinichthys multiradiatus*. *Ethology Ecology & Evolution* **13**:331-339.

MacVean CM (1987) Ecology and management of the Mormon cricket, *Anabrus simplex* Haldeman. In: Capinera JL (ed) *Integrated Pest Management on Rangeland: A Shortgrass Prairie Perspective*, Westview Press: Boulder, Colo, 116-136.

MacVean CM (1990) Mormon crickets: a brighter side. *Rangelands* **12**:234-235.

Maddison WP (1997) Gene trees in species trees. *Systematic Biology* **46**:523-536.

Maddison WP, Maddison DR (2004) *Mesquite: a modular programming system for evolutionary analysis*. v.1.0. http://mesquite-project.org.

Madsen DB (1989) A grasshopper in every pot. *Natural History* **7/89**:22-25.

Madsen T, Shine R, Olsson M, Wittzell H (1999) Restoration of an inbred adder population. *Nature* **402**:34-35.

Mandelson TC, Shaw KL (2005) Rapid speciation in an arthropod. *Nature* **433**:375-376.

Matsuura K, Kuno E, Nishida T (2002) Homosexual tandem running as selfish herd in *Reticulitermes speratus*: novel antipredatory behaviour in termites. *Journal of Theoretical Biology* **214**:63-70.

Mésochina P, Bedin E, Ostrowski S (2003) Reintroducing antelopes into arid areas: lessons learnt from the oryx in Saudi Arabia. *Comptes Rendus Biologies* **326**:S158–S165.

Merola M (1994) A reassessment of homozygosity and the case for inbreeding depression in the cheetah (*Acinonyx jubatus*): implications for conservation. *Conservation Biology* **8**:961-971.

Mills HB, Hitchcock OB (1938) Control of Mormon crickets in Montana. *Montana Extension Service in Agriculture and Home Economics* Bulletin No. 160.

Montealegre-Z F, Mason AC (2005) The mechanics of sound production in *Panacanthus pallicornis* (Orthoptera: Tettigoniidae: Conocephalinae): the stridulatory motor patterns. *The Journal of Experimental Biology* **208**:1219-1237.

Moran MD (2003) Arguments for rejecting the sequential Bonferroni in ecological studies. *OIKOS* **100**:403-405.

Mullis KB, Faloona FA (1987) Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods in Enzymology* **155**:335-50.

Mullis KB, Faloona FA, Scharf S, Saiki RK, Horn G, Erlich HA (1986) Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. *Cold Spring Harbor Symposia on Quantitative Biology*

New York Times (1990) War on six legs: Nevada's cricket invasion. Sunday, April 8.

Nice CC, Anthony N, Gelembiuk G, Raterman D, French-Constant R (2005) The history and geography of diversification within the butterfly genus *Lycaeides* in North America. *Molecular Ecology* **14**:1741-1754.

Nichols RA (2001) Gene trees and species trees are not the same. *Trends in Ecology and Evolution* **16**:358-364.

Nichols RA, Bruford MW, Groombridge JJ (2001) Sustaining genetic variation in a small population: evidence from the Mauritius kestrel. *Molecular Ecology* **10**:593-602.

O'Neill KM, Turnbow JH (1999) Thermoregulation and temperature-dependent *Beauveria bassiana* mycosis in Mormon crickets (invited talk in Section Symposium: "Life on the Edge") Entomological Society of America, National Meetings, Atlanta, Georgia, December 1999.

Orange DI, Riddle BR, Nickle DC (1999) Phylogeography of a wide-ranging desert lizard, *Gambelia wislizenii* (Crotaphytidae). *Copeia* **2**:267-273.

Pace M (2003) Mormon cricket control. USU Extension Agent, Millard County. http://extension.usu.edu/hoppers/htmfiles/

Page RDM, Holmes EC (2002) Molecular evolution: a phylogenetic approach. Blackwell Science Ltd., Oxford.

Pál C, Miklós I (1999) Epigenetic inheritance, genetic assimilation and speciation. *The Journal of Theoretical Biology* **200**:19-37.

Panhuis TM, Butlin R, Zuk M, Tregenza T (2001) Sexual selection and speciation. *Trends in Ecology and Evolution* **16**:364-371.

Perkins A, Fitzgerald JA, Moss GE (1995) A comparison of LH secretion and brain estradiol receptors in heterosexual and homosexual rams and female sheep. *Hormones and Behaviour* **29**:31-41.

Posada D, Crandall KA (1998) Modeltest: testing the model of DNA substitution. *Bioinfomatics* **14**:817-818.

Prestwich KN, Lenihan KM, Martin DM (2000) The control of carrier frequency in cricket calls: a refutation of the subalar-tegminal resonance/auditory feedback model. *The Journal of Experimental Biology* **203**:585-596.

Price EO, Katz LS, Wallach SJR, Zenchalk JJ (1988) The relationship of male-male mounting to the sexual preferences of young rams. *Applied Animal Behaviour Science* **21**:347-352.

Queller DC (1997) Why do females care more than males? *Proceedings of the Royal Society of London* B **264**:1555-1557.

Raufaste N, Rousset F (2001) Are partial Mantel tests inadequate? *Evolution* **55**:1703-1705.

Raymond M, Rousset F (1995) GENEPOP (version 3.4): population genetics software for exact tests and ecumenicism. *Journal of Heredity* **86**:248-249.

Redak RA, Capinera JL, Bonham CD (1992) Effects of sagebrush removal and herbivory by Mormon crickets (Orthoptera: Tettigoniidae) on understory plant biomass and cover. *Ecological Entomology* **21**:94-102.

Remington CL (1968) Suture-zones of hybrid interaction between recently joined biotas. *Evolutionary Biology* **2**:321-428.

Rice G, Anderson C, Risch N, Ebers G (1999) Male homosexuality: absence of linkage to microsatellite markers at Xq28. *Science* **284**:665-667.

Rice WR, Hostert EE (1993) Laboratory experiments on speciation: what have we learned in 40 years? *Evolution* **47**:1637-1653.

Riddle BR (1996) The molecular phylogeographic bridge between deep and shallow history in continental biotas. *Trends in Ecology and Evolution* **11**:207-211.

Riddle BR, Hafner DJ, Alexander LF (2000) Phylogeography and systematics of the *Peromyscus eremicus* species group and the historical biogeography of north American warm regional deserts. *Molecular Phylogenetics and Evolution* **17**:145-160.

Ritchie MG, Butlin RK, Hewitt GM (1987) Causation, fitness effects and morphology of macropterism in *Chorthippus parallelus* (Orthoptera: Acrididae). *Ecological Entomology* **12**:209-218.

Ritchie MG (1991) Female preference for 'song races' of *Ephippiger ephippiger* (Orthoptera: Tettigoniidae). *Animal Behaviour* **42**:518-520.

Ritchie MG, Couzin ID, Snedden WA (1995) What's in a song? Female bushcrickets discriminate against the song of older males. *Proceedings of the Royal Society of London* B **262**:21-27.

Ritchie MG, Kidd DM, Gleason JM (2001) Mitochondrial DNA variation and GIS analysis confirm a secondary origin of geographical variation in the bushcricket *Ephippiger ephippiger* (Orthoptera: Tettigonioidea). *Molecular Ecology* **10**:603-611.

Ritchie MG, Racey SN, Gleason JM, Wolff K (1997) Variability of the bushcricket *Ephippiger ephippiger*: RAPDs and song races. *Heredity* **79**:286-294.

Ritchie MG, Sunter D, Hockham LR (1998) Behavioral components of sex role reversal in the Tettigoniid bushcricket *Ephippiger ephippiger*. *The Journal of Insect Behaviour* **11**:481-491.

Rocha LA, Robertson DR, Rocha CR, Van Tassell JL, Craig MT, Bowen BW (2005) Recent invasion of the tropical Atlantic by an Indo-Pacific coral reef fish. *Molecular Ecology* **14**:3921-3928.

Roff DA, Mosseau TA, Howard DJ (1999) Variation in genetic architecture of calling song among populations of *Allonemobius socius*, *A. fasciatus*, and a hybrid population: drift or selection? *Evolution* **53**:216-224.

Römer H, Bailey W (1998) Strategies for hearing in noise: peripheral control over auditory sensitivity in the bushcricket *Sciarasaga quadrata* (Austrosaginae: Tettigoniidae). *The Journal of Experimental Biology* **201**:1023-1033.

Roselli CE, Larkin K, Resko JA, Stellflug JN, Stormshak F (2004) The volume of a sexually dimorphic nucleus in the ovine medial preoptic area/anterior hypothalamus varies with sexual partner preference. *Endocrinology* **145**:478-483.

Rosenburg NA, Nordborg M (2003) Genealogical trees, coalescent theory and the analysis of genetic polymorphisms. *Nature* **3**:380-390.

Rousset F (2002) Partial Mantel tests: reply to Castellano and Balletto. *Evolution* **56**:1874-1875

Rozas J, Sanchez-DelBarrio JC, Messeguer X, Rosas R (2003) DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics* **19**:2496-2497.

Saccheri IJ, Barkefield PM, Nichols RA (1996) Severe inbreeding depression and rapid fitness rebound in the butterfly *Bicyclus anynana* (Satyridae). *Evolution* **50**:2000-2013.

Sagvik J, Uller T, Olsson M (2004) Outbreeding depression in the common frog, *Rana temporaria*. *Conservation Genetics* **6**:205-211.

Scheuber H, Jacot A, Brinkhof MWG (2003a) Condition dependence of a multicomponent sexual signal in the field cricket *Gryllus campestris*. *Animal Behaviour* **65**:721-727.

Scheuber H, Jacot A, Brinkhof MWG (2003b) The effect of past condition on a multicomponent sexual signal. *Proceedings of the Royal Society of London* B **270**:1779-1784.

Schlötterer C (2000) Evolutionary dynamics of microsatellite DNA. Chromosoma **109**:365-371.

Schmitt T, Cizek O, Konvicka M (2005) Genetics of a butterfly relocation: large, small and introduced populations of the mountain endemic *Erebia epiphron silesiana*. *Biological Conservation* **123**:11-18.

Schneider S, Roessli D, Excoffier L (2000) *Arlequin ver. 2.000: A software for population genetics data analysis*. Genetics and Biometry Laboratory, University of Geneva, Switzerland.

Schug MD, Hutter CM, Noor MAF, Aquadro CF (1998) Mutation and evolution of microsatellites in *Drosophila melanogaster*. *Genetica* **102-3**:359-367 Sp. Iss.

Schul J, Patterson AC (2003) What determines the tuning of hearing organs and the frequency of calls? A comparative study in the katydid genus *Neoconocephalus* (Orthoptera, Tettigoniidae). *The Journal of Experimental Biology* **206**:141-152.

Schweis GG, Burge LM, Shocren GM (1939) Mormon cricket control in Nevada, 1936-1938. *Nevada State Department of Agriculture Bulletin no. 2* (Reno, Nevada).

Sears FW, Zemansky MW (1964) University Physics. Third edition complete. Addison Wesley.

Seidelmann K, Ferenz H-J (2002) Courtship inhibition pheromone in desert locusts, *Schistocerca gregaria*. *Journal of Insect Physiology* **48**:991-996.

Shapiro AM (1976) Seasonal Polyphenism. *Evolutionary Biology* **9**:259-333.

Shine R, Harlow P, Lemaster MP, Moore IT, Mason RT (2000) The transvestite serpent: why do male garter snakes court (some) other males? *Animal Behaviour* **59**: 349-359.

Simon C, Frati F, Beckenbach A, Crespi B, Liu H, Flook P (1994) Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Annals of the Entomological Society of America* **87**:651-701.

Simmons LW (1995) Correlates of male quality in the field cricket *Gryllus campestris* L.: age, size, and symmetry determine pairing success in field populations. *Behavioural Ecology* **6**:376-381.

Simmons LW, Bailey WJ (1990) Resource influenced sex roles of *Zaprochiline* tettigoniids (Orthoptera: Tettigoniidae). *Evolution* **44**:1853-1868.

Simmons LW, Ritchie MG (1996) Symmetry in the songs of crickets. *Proceedings of the Royal Society of London* B **263**:305-311.

Simpson SJ, Despland E, Hägele BF, Dodgson T (2001) Gregarious behavior in desert locusts is evoked by touching their back legs. *Proceedings of the National Academy of Sciences of the USA* **98**:3895-3897.

Slate J, David P, Dodds KG, Veenvliet BA, Glass BC, Broad TE, McEwan JC (2004) Understanding the relationship between the inbreeding coefficient and multilocus heterozygosity: theoretical expectations and empirical data. *Heredity* **93**:255-265.

Slatkin M, Maddison WP (1989) A cladistic measure of gene flow inferred from the phylogenies of alleles. *Genetics* **123**:603-613.

Small MP, Stone KD, Cook JA (2003) American marten (*Martes americana*) in the Pacific Northwest: population differentiation across a landscape fragmented in time and space. *Molecular Ecology* **12**:89-103.

Smithsonian video collection (1992) The history of PCR (RU 9577) http://www.si.edu/archives/ihd/videocatalog/9577.htm.

Snodgrass RE (1905) The coulee cricket of central Washington (*Peranabrus scabricollis* Thomas). *Journal of the New York Entomological Society* **13**:74-83.

Sokal RR, Rohlf FJ (1969) *Biometry*. W. H. Freeman and Company: San Francisco, CA.

Sorenson CJ, Jeppson LR (1940) Some insect pests of farm crops in the Juniper-Pinion belt of Utah. *Proceedings of the Utah Academy of Sciences Arts and Letters* **17**:49-52.

Spielman D, Brook BW, Frankham R (2004) Most species are not driven to extinction before genetic factors impact them. *Proceedings of the National Academy of Sciences of the USA* **101**:15261-15264.

Starkey DE, Shaffer HB, Burke RL, Forstner MRJ, Iverson JB, Janzen FJ, Rhodin AGJ, Ultsch GR (2003) Molecular systematics, phylogeography, and the effects of Pleistocene glaciation in the painted turtle (*Chrysemys picta*) complex. *Evolution* **57**:119-128.

Stephen RO, Hartley JC (1995) Sound production in crickets. *The Journal of Experimental Biology* **198**:2139-2152.

Stockwell CA, Mulvey M, Vinyard GL (1996) Translocations and the preservation of allelic diversity. *Conservation Biology* **10**:1133-1141.

Storz JF, Beaumont MA (2002) Testing for genetic evidence of population expansion and contraction: an empirical analysis of microsatellite DNA variation using a hierarchical Bayesian model. *Evolution* **56**:154-166.

Swabb DF, Hofman MA (1990) An enlarged superchiasmatic nucleus in homosexual men. *Brain Research* **537**:141-148.

Swenson NG, Howard DJ (2004) Do suture zones exist? *Evolution* **58**:2391-2397.

Swofford DL (1998) *PAUP*: Phylogenetic Analysis Using Parsimony and other methods*. Beta version 4.0b10. Sinauer Associates, Sunderland, Mass.

Sword GA (2002) A role for phenotypic plasticity in the evolution of aposematism. *Proceedings of the Royal Society of London* B **269**:1639-1644.

Sword GA, Simpson SJ (2000) Is there an intraspecific role for density-dependent colour change in the desert locust? *Animal Behaviour* **59**:861-870.

Sword GA (2005) Local population density and the activation of movement in migratory band-forming Mormon crickets. *Animal Behaviour* **69**:437-444.

Sword GA, Lorch PD, Gwynne DT (2005) Migratory bands give crickets protection. *Nature* **433**:703.

Technelysium Pty. Ltd. *Chromas 2.12*. Copyright 1998-2001. PO Box 247, Helensvale, Queensland 4212, Australia.

Templeton AR (1998) Nested clade analyses of phylogeographic data: testing hypotheses about gene flow and population history. *Molecular Ecology* **7**:381-397.

Templeton AR (2004) Statistical phylogeography: methods of evaluating and minimizing inference errors. *Molecular Ecology* **13**:789-809.

Thomas LK, Manica A (2005) Intrasexual competition and mate choice in assassin bugs with uniparental male and female care. *Animal Behaviour* **69**:275-281.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997). The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **24**: 4876-4882.

Tomkins JL, Brown GS (2004) Population density drives the local evolution of a threshold dimorphism. *Nature* **431**:1099-1103.

Tregenza T (2002) Divergence and reproductive isolation in the early stages of speciation. *Genetica* **116**:291-300.

Tregenza T, Pritchard VL, Butlin RK (2000) The origins of premating reproductive isolation: Testing hypotheses in the grasshopper *Chorthippus parallelus*. *Evolution* **54**:1687-1698.

——— (2002) The origins of postmating reproductive isolation: testing hypotheses in the grasshopper *Chorthippus parallelus*. *Population Ecology* **44**: 137-144.

Trivers RL (1972) Parental investment and sexual selection. In: Sexual selection and the descent of man 1871-1971 (Ed. B. Campbell) Pp: 136-179. Chicago: Aldine.

Ueckert DN, Hansen RN (1970) Seasonal dry-weight composition in diets of Mormon crickets. *Journal of Economic Entomology* **63**:96-98.

Waddington CH (1953) Genetic assimilation of an acquired character. *Evolution* **7**:118-126.

Wakeland C (1959) Mormon crickets in North America. USDA Technical Bulletin 1202.

Walker TJ (1974) Effects of temperature, humidity, and age on stridulatory rates in *Atlanticus* spp. (Orthoptera: Tettigoniidae: Decticinae). *Annals of the Enttomological Society of America* **68**:607-611.

Webb SA, Miller RR (1998) *Zoogoneticus tequila*, a new Goodeid fish (Cyprinodontiformes) from the Ameca drainage of Mexico, and a rediagnosis of the genus. *Occasional Papers of the Museum of Zoology, University of Michigan* **725**:1–23.

Weber JL, Wong C (1993) Mutation of human short tandem repeats. *Human Molecular Genetetics* **2**:1123-1128.

Weir JT, Schluter D (2004) Ice sheets promote speciation in boreal birds. *Proceedings of the Royal Society of London* B **271**:1881-1887.

West-Eberhard MJ (1983) Sexual selection, social competition, and speciation. *Quarterly Reviews in Biology* **58**:155-183.

Williams M, Dunkerley D, De Deckker P, Kershaw P, Chappell J (1998) Quaternary Environments (Arnold, London).

www.utah.com/visitor/state_facts/symbols.htm (2005) Utah state symbols. Courtesy of the Governor's office.

Yamamoto D, Ito H, Fujitani K (1996) Genetic dissection of sexual orientation: behavioral, cellular, and molecular approaches in *Drosophila melanogaster*. *Neuroscience Research* **26**:95-107.

Zenchak JJ, Anderson GC, Schein MW (1981) Sexual partner preferences of adult rams (*Ovis aries*) as affected by social experiences during rearing. *Applied Animal Ethology* **7**:157-167.

Zhang D-X, Yan L-N, Ji Y-J, Kang L, Hewitt GM, Huang Z-S (2003) Isolation, characterization and cross-species amplification of eight microsatellite DNA loci in the migratory locust (*Locust migratoria*). *Molecular Ecology Notes* **3**:483-486.

Zuk M, Simmons LW, Cupp L (1993) Calling characteristics of parasitized and unparasitized populations of the field cricket *Teleogryllus oceanicus*. *Behavioural Ecology and Sociobiology* **33**:339-343.

Zuk M, Rotenberry JT, Simmons LW (1998) Calling songs of field crickets (*Teleogryllus oceanicus*) with and without phonotactic parasitoid infection. *Evolution* **52**:166-171.