PATTERNS OF SPECIATION AMONG ALLOPATRIC *Drosophila mettleri* POPULATIONS

by

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DEDICATION

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TABLE OF CONTENTS

TABLE OF CONTENTS - C*ontinued*

LIST OF ILUSTRATIONS

LIST OF TABLES

ABSTRACT

Sonoran Desert *Drosophila mettleri* breeds in soil soaked by the necrotic cacti juices from saguaro (*Carnegiea gigantea*) and cardon (*Pachycereus pringlei*). An isolated population on Santa Catalina Island, 300 kilometers NW of the Sonoran Desert limit, was discovered breeding in several *Opuntia* cacti species. Host shifts are associated with the speciation process in phytophagous insects. I tested for evidence of premating isolation, postmating isolation, and ecological differences among allopatric populations of *Drosophila mettleri* using a variety of approaches. No sexual isolation was detected. However, *Drosophila mettleri* from Santa Catalina Island shows significant behavioral and physiological differences compared with Sonoran Desert populations. Furthermore, *Drosophila mettleri* from Santa Catalina Island was significantly genetically differentiated from all other populations in the study. Finally, I observed sufficiently significant F_1 male sterility in crosses involving the Santa Catalina Island population to consider it indicative of early postzygotic isolation.

CHAPTER 1: GENERAL INTRODUCTION

Speciation is the process by which new species arise. However, what is a species? Several systematic and evolutionary biologists have suggested that we need to answer this fundamental question in evolutionary biology before we can investigate the process of species formation (Templeton 1989, Berlocher 1998). The morphological species concept was perhaps the most influential species definition during the nineteen-century. However, after the modern synthesis, several concepts of species have appeared. Some of the most important concepts of species in the literature are the recognition (Dobzhansky 1970), the evolutionary (Wiley 1978), the cohesion (Templeton 1989), the phylogenetic or character-based (Cracraft 1989), the genealogical (Baum and Shaw1995), and the genotypic cluster (Mallet 1995) concepts. Nevertheless, Mayr (1963) proposed the Biological Species Concept (BSC) which became the most influential species definition over the last decades, particularly for sexually reproducing organisms. Mayr's definition is "[G]roups of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups" Obviously, the BSC has some weakness. The "interbreeding" criterion is useless for asexual organisms or extinct forms (Campbell 1993). In addition, BSC is inadequate if we cannot know if two geographically segregate populations have the potential to interbreed in nature (Mayr 1996).

Subsequently, the speciation process as the evolution of a complete lack of interbreeding between individuals from diverged populations remains the major problem

in evolutionary biology. Several speciation modes have been proposed to explain elimination/reduction of migration and/or gene flow between populations (Bush 1969; Via 2001). However, the allopatric speciation mode which is the evolution of genetic reproductive barriers between populations that are geographically separated by physical barriers has become thought of as the prevalent mode of speciation in animals (Mayr 1963; Carson 1975**;** Futuyma 1998; Coyne and Orr 1989). Genetic differences between allopatric populations affecting morphology, physiology and/or behavior are considered to be caused by one or a combination of mutation, genetic drift, and natural selection (Muller 1942; Mayr 1942, 1963; Endler 1986**).** Under the BSC, the final stage of the speciation is the evolution of prezygotic and/or postzygotic isolation between allopatric populations (Mayr 1963; Orr 2001). In fact, Coyne & Orr (1997) suggested that prezygotic isolation evolves faster than postzygotic isolation, and both types of isolation increase with divergence time between them.

To study early events of the speciation process, it is important to identify early stages of morphological, physiological and/or behavioral divergence among allopatric populations that may reflect genetic changes leading to reproductive isolation. In fact, for phytophagous insects, behavioral and physiological adaptations caused by host shifts have been proposed as among the first adjustments that allopatric populations experience on the way to the speciation process (Schultz 1988; Jaenike and Holt 1991).

The North American Sonoran Desert provides an exceptional ecosystem for addressing questions about the allopatric mode of speciation in terrestrial organisms,

especially in small species in the class Insecta. Four species in family Drosophilidae colonized the Sonoran Desert independently (Fogleman *et al.* 1981). These *Drosophila* have been adapted to use highly toxic necroses produced by columnar cacti which are lethal to Non-Sonoran Desert *Drosophila* (Kircher *et al.* 1967; Kircher 1982; Frank and Fogleman 1992). One of these cactophilic species is *D. mettleri*, a species in the eremophila species complex of the mulleri subgroup of the repleta group (Heed 1977). The typical host cacti of *D. mettleri* are the cardon cactus (*Pachycereus pringlei*) mainly in the Baja California peninsula and the saguaro cactus (*Carnegiea gigantea*) in the continental part of the Sonoran Desert (Heed 1977, 1982; Heed and Mangan 1986). *Drosophila mettleri* and D. *nigrospiracula,* another of the four endemic species, are similar in appearance and share the same feeding niche. However, *D. mettleri* is one of the few true soil breeders in the Drosophilidae family. Females oviposit in the soil soaked by necrotic juices from its large columnar hosts where the high alkaloid concentration is lethal to other desert *Drosophila* (Heed 1977, 1978, 1982; Fogleman *et al*. 1982; Fogleman and Williams 1987). On the other hand, an isolated *D. mettleri* population, outside the limits of the Sonoran Desert, was found on Catalina Island in the Southern California coast where columnar cacti are not present (Heed 1989). On this island, *D. mettleri* shifted hosts and now utilizes necrotic tissue of *Opuntia demissa, O. oricola* and *O. littoralis* as larval niche (Heed 1989; Castrezana, unpublished data). However, decayed *Opuntia* plants do not contain the same toxic allolechemical deterrents normally present in Sonoran Desert columnar cacti (Fogleman *et al.*1982; Danielson *et al.*. 1997; Fogleman and Danielson 2001).

My research assumes the BSC as the most appropriate way to define a species. Then chemical, morphological, and resource availability differences among geographic hosts instigate questions about a potential incipient speciation process. I used the cactophilic *Drosophila mettleri* to examine whether or not early stages of the speciation process can be identified. I have divided my research in three chapters.

In Chapter Two, I look at the degree of prezygotic or sexual isolation in four allopatric populations of *D. mettleri* using four types of choice test to obtain reproductive isolation indices. In Chapter Three, I devote my research to the ecology, reproductive output, and sexual behavior in four allopatric populations of *D. mettleri*. I ask the degree to which populations differ in reproductive output, the genetic basis of these differences, and if these differences in reproductive output are associated with differences observed in copulation duration. I also speculate on how host shift may affect the divergence of the peripheral population. Finally, in Chapter Four I center my attention on the population genetic differentiation of twelve allopatric populations of *D. mettleri* using alloenzyme electrophoresis and mitochondrial DNA sequences, and in the postzygotic isolation of four allopatric populations of *D. mettleri* using development time, sex ratio and male sterility as indicators. I ask the degree of genetic differentiation among populations, and then search for a potential evidence of postzygotic isolation. Lastly, I ask if evidence of postzygotic isolation is associated with the degree of genetic differentiation.

CHAPTER 2: SEXUAL ISOLATION OR MATING PROPENSITY DIFFERENCES AMONG ALLOPATRIC *Drosophila mettleri* POPULATIONS

Abstract

Drosophila mettleri is endemic to the Sonoran Desert of North America breeding in soil soaked by the juices of necrotic cacti. Saguaro (*Carnegiea gigantea)* and cardón (*Pachycereus pringlei)* are the usual host cacti in Mexico and Arizona. Populations of *D. mettleri* are associated with different host cacti, however, in other parts of the species' range and show significant local genetic differentiation, especially when geographical isolation is coupled with host shifts. We tested for evidence of sexual isolation among allopatric populations of *D. mettleri* using a variety of approaches. Populations exhibited significant differences in mating propensity, which translated into significant deviations from random mating. While in some cases these deviations were consistent with sexual isolation, in others, negative assortative mating was observed.

Introduction

A major problem in evolutionary biology is understanding the way in which new species form. While there is renewed interest in defining the importance of sympatric speciation (Bush 1969, 1975; Berlocher 1998; Kondrashov *et al.*1998; Via 2001), speciation in allopatry appears to be the most common process by which speciation occurs (Dobzhansky 1941; Mayr 1963; Carson 1975; Templeton 1980; Coyne and Orr 1989, 1997). This being the case, the ability to study early events in speciation depends upon the identification of populations of the same species that are likely to be at early

stages of divergence. The likelihood of divergence is increased if populations are geographically separated, utilize different resources, and/or are exposed to different environmental factors that could act as potential forces of selection and drive differentiation (Schluter 2001).

Drosophila mettleri is a cactophilic species found in the Sonoran Desert of North America, where it breeds in the soil soaked by necrotic columnar cacti of several different species (Heed 1977, 1978; Markow *et al.* 1983). Their geographic range and primary local host cacti are shown in Figure 1. Because the larger cacti, such as cardón (*Pachycereus pringlei*) and saguaro (*Carnegiea gigantea*) provide greater quantities of necrotic juice than most organ pipe (*Stenocereus thurberi*) or senita (*Lophocereus schottii*), larval *D. mettleri* are most often associated with necroses of saguaro or cardón in these regions (Heed 1977, 1982; Heed and Mangan 1986). On the other hand, *D. mettleri* also has been found on Santa Catalina Island (Heed 1989), off the coast of southern California, where no columnar cacti are found and where adults have been reared from necrotic pads of *Opuntia littoralis* (Castrezana, unpublished data), a prickly pear cactus found on the island.

Genetic studies of *D. mettleri* from across its range reveal significant differentiation only between the Santa Catalina Island population and the other localities (Hurtado *et al.*2004; Markow *et al.*2002). Among strains from t he Baja California peninsula and the Sonoran mainland or Arizona, where the Sea of Cortez should serve to restrict gene flow and where flies shift host plants and experience different climatic

conditions on either side of the Sea, genetic differentiation, when it is detected, is only minimal. Furthermore, there is no evidence of postzygotic barriers promoting isolation among the different geographic host strains of *D. mettleri* (Markow *et al.*2002; chapter 4). In another desert endemic cactophilic *Drosophila*, *D. mojavensis*, that occupies the same range as *D. mettleri,* the population from Santa Catalina Island does exhibit some prezygotic isolation from mainland populations (Markow and Hocutt 1998; Reed and Markow 2004), suggesting that Santa Catalina Island *D. mettleri* be further examined for evidence of similar isolating mechanisms.

Coyne and Orr (1989, 1997) have examined patterns of reproductive isolation among *Drosophila*, which have already become different species and found evidence that premating isolation may precede postmating isolation during the speciation process. Because the studies they reviewed dealt with species that have already formed, it is unclear, when considering populations that have not yet achieved the status of species, at what point during the differentiation process sexual isolation appears and whether it typically precedes postzygotic isolation. Populations of *Drosophila mettleri* provide an opportunity to ask if behavioral isolation precedes postmating isolation during genetic differentiation. In the present study, we ask if there is evidence of behavioral isolation among the geographic populations of *D. mettleri* and if it exists, if it is most pronounced between populations exhibiting the greatest degree of genetic differentiation, such as those from Santa Catalina Island compared to those from Sonora. We use a range of experimental designs not only to reveal patterns of nonrandom mating, but also to ask if

the behavioral isolation observed is a function of choice or it reflects differences in mating propensity by males and females of the different populations.

Studies of sexual isolation in *Drosophila* have employed a wide range of experimental designs (Malogolowkin-Cohen *et al.* 1965; Markow 1980; Zouros and D'Entremont 1980). These tests typically are referred to as "choice tests" although the degree to which they actually measure "choice" has been questioned (Marin 1991; Casares *et al.*1998). Nonetheless, they represent standard tests for such studies and provide accessible and useful measures of departures from random mating. In the "no choice" design, a single female and single male are placed in a vial. In "female choice" tests, a single female or group of females of the same strain, are placed with two males or two groups of males representing their own and another strain. In "multiple choice" tests, equal numbers of females and males from two strains are placed together. Random mating is usually tested for by direct observation, often with either wing clipping or colored dust to distinguish flies of different strains. In some cases, however, flies are left together for 24 hours and departures from random mating are scored by progeny tests or dissection of females to detect insemination. A potential problem with this last approach is the possibility of confounding premating isolation with postmating-prezygotic interactions, which may underestimate the actual number of matings that took place (Gilbert and Starmer 1985). Therefore, in the present study, courtship and mating were observed directly.

A problem with using choice tests alone to estimate isolation is the inability to infer anything about the processes leading to nonrandom mating, should it be detected. Male courtship propensity or vigor exemplified by short courtship latencies, and female propensity, characterized by rapid receptivity, can conspire to create departures from random mating having nothing to do with choice or preference (Casares *et al.* 1998; Rolán-Alvarez and Caballero 2000). In our study we first characterize mating propensity in both sexes of all strains in order to detect differences that would predict specific vigor-based patterns of nonrandom mating in choice tests and to separate them from the effects of preferences. We then employ a series of choice tests (female choice, male choice, multiple choice) to evaluate departures for random mating as well as deviations from random mating based upon propensity alone.

We used four strains of *D. mettleri*, derived from collections at geographically distant locations (Figure 1) for which levels of genetic differentiation have been previously determined (Hurtado et al 2004; Markow et al 2002; Chapter 3) to examine sexual isolation. *Drosophila mettleri* from Santa Catalina Island, California, exhibit the greatest genetic differentiation from the other populations, leading to the prediction that sexual isolation, if observed, should be greatest between this and the other populations.

Material and methods

Collection and handling of stocks**:** Four strainsof *D. mettleri* were selected to use in this experiment due to their degree of geographic separation. Collection sites and dates were as follows: a) Santa Catalina Island, CA (3/97), designated “CAT”; b) Superstition

Mountains, AZ (3/97), designated “SUP”; c) Loreto, Baja California, Mexico (11/96), designated “LO”; and d) from Guaymas, Sonora, Mexico (11/96), designated “GYM”. Each strain was founded from a multiple female collection (n>40) and maintained in large numbers in half-pint bottles on potato cactus medium (Castrezana 1997). Virgin flies were separated by sex under CO_2 and stored in standard banana food vials until five to six days old at which time they were used in behavioral tests.

Behavioral tests: Four different types of behavioral tests were conducted: (1) no-choice tests of mating propensity, (2) male choice tests, (3) female choice tests, and (4) multiple choice tests.

No-choice tests: Four control lines and 12 inter-strain combinations were tested. A mature female and male were aspirated into a vial and observed until copulation occurred or 15 minutes had elapsed. In the no choice tests, times, in seconds, until the following behavioral landmarks were observed were recorded: male courtship latency (time from introduction until first chasing, wing vibration or licking); female receptivity (female wing spreading to allow mounting and intromission); overall mating speed (time from introduction until successful copulation). At least 40 pairs of flies were observed for each combination. Also, data for pairs not courting or mating were recorded to obtain percent of successful courtship. The proportion of pairs not reaching a particular landmark was also determined.

Female choice tests: Twelve combinations were tested. A virgin female was aspirated into a vial with two virgin males; one from her own and one from a different

population. Males had been dusted lightly with radiant colors from Magruder Color Co (Alameda, CA) and allowed to clean themselves for two hours before the experiment. Colored dusts were tested prior to the study and found to have no influence on the experimental outcomes. The strain of the first male to court and the male to mate were recorded. At least 60 females were used for each combination.

Male choice test: Twelve combinations were tested. A virgin male was aspirated into a vial with two virgin females; one from his own and one from a different population. Females were lightly treated with colored dust. The strain of the first female courted and the female mating were recorded and analyzed using chi-square tests. At least 60 males were used for each combination.

Multiple choice test: Six combinations were tested. In a standard Plexiglas mating arena (400 cm^2) two populations were tested per combination. For each population, five females and five males (previously dusted with radiant colors) were introduced simultaneously into the mating arena. Combinations of strains mating were recorded. Chambers were observed for 20 minutes. At least 20 arenas per combination were tested.

Statistical analyses: Courtship latency, receptivity, and overall mating speed were subjected to factorial ANOVA with male and female strain as the two factors. Departures from random mating in the choice tests were analyzed by Chi-square tests. The foregoing analyses were conducted using JMP software version 4.0.4 (A business unit of SAS, SAS Institute Inc., 2001). In addition, isolation indices were calculated for all choice tests. The formulae used were

Female choice tests: I = 1 – (frequency of heterospecific matings/frequency of homospecific matings) (Coyne and Orr 1989)

Male choice test: I = 1 – (frequency of heterospecific matings/frequency of homospecific matings) (Coyne and Orr 1989)

Multiple choice test: I = ((frequency of homospecific matings)-(frequency of heterospecific matings))/N (Malogolowkin-Cohen *et al.* 1965)

Standard errors for all choice test were calculated according to:

s.e. = $\sqrt{(1-I^2)/N}$ (Malogolowkin-Cohen *et al.* 1965)

Results

No choice tests. Before providing flies from different geographic strains a "choice" of mates, the relative mating propensity of males (courtship latency) and females (receptivity latency) from each strain was examined under "no choice" conditions. Of the 100 pairs observed for each of the four-intrapopulation groups, not all males courted before the cut-off point and not all courted females were receptive. Male courtship, therefore, doesn't guarantee that mating eventually will occur, as females may not be receptive. For example, during the observation period, 90 % of pairs of the GYM strain exhibited courtship, and yet females were receptive to courting males in only 65% of pairs. Slightly less than 80 % of courted females of the LO and SUP strains were receptive during the observation period (Figure 2).

Male courtship latency, time until female receptivity, and overall mating speed were recorded for all pairs in which these behavioral landmarks were observed (Table 1).

ANOVA revealed a significant effect of male but not female strain on male courtship latency (Table 2a). Female receptivity was significantly influenced by both female strain and male strain (Table 2b), as was the overall time from introduction of the pairs until mating was observed.

If the progress and success of between-strain pairings are a function only of male courtship propensity, the above observations would predict that any interpopulation pairing involving a GYM or LO male would be exhibit slower male courtship latencies and or mating success. If factors other than, or in addition to, male propensity, such as male discrimination between females based upon female strain and / or preference for particular female type in order to initiate or continue courtship, different patterns, such as dependence on female type, are predicted.

The outcome of no-choice, between strain pairings was measured in two ways: the proportion of pairs achieving a particular courtship landmark such as courtship or copulation, and the actual latencies recorded for male courtship or female receptivity. The proportions of interpopulation pairs reaching the courtship and copulation landmarks are shown in Figure 3a-f. Within-strain control values are presented on the same figures as the corresponding interstrain data in order to more easily visualize the nature and degree of any difference observed between intra and interstrain pairs. In the three cases where CAT individuals were present, CAT x SUP (a), CAT x GYM (e), and CAT x LO (f), the proportion mating is intermediate between the proportions for the two within

strain pairings. In general, males and females from the GYM strain tended to mate more readily with flies from strains other than their own.

Male latency, female receptivity and overall mating speeds for between-strain pairings are shown in Table 1. The most striking features of the pairings between strains is the significant, exaggerated latency of LO males to initiate courtship when females are not from their population, which resulted in a significant delay in the overall mating speeds in these pairings.

Choice Tests: tests for nonrandom mating: The differences in mating propensity revealed above enable us to make specific predictions about the outcomes of "choice" tests. If results of choice tests are a function of differences in general mating propensity rather than a true preference for, or discrimination against, potential mates from a given strain, certain patterns should be observed in choice tests. For example, if mating propensity (male vigor or female receptivity) is the sole factor in determining any deviation from random mating among flies of two different strains, there should be a higher than expected number of matings between males of the strain showing the greatest vigor and females of the strain with the quickest receptivity. Males from strains showing slow courtship latencies and females from strains having low receptivity would not be mating as often as males that are quick to court and females that are quick to mate. The observation that LO and GYM males are slower than males of the other two strains to begin courting, predicts that they will be at a disadvantage in female choice tests, especially when females are from the somewhat more receptive CAT or LO strains

(Table 1). In male choice tests, an excess of matings is predicted to occur with the strain of female that exhibits faster receptivity more frequently.

During male choice tests, the first female courted by the male as well as the female that mated were recorded (Table 3). In partitioning the observations in this way, we could observe whether the first female courted was actually the female the male subsequently mated with. In no case was there a significant deviation from random courtship (Table 3a). Of those combinations which approached statistical significance with respect to first female courted (CAT males with SUP females, GYM males with CAT and with SUP females, and LO males with GYM females), the tendency was to court strange females first. With respect to actual copulation, on the other hand, three significant deviations from random mating were observed, as reflected in both X^2 and isolation index values (Table 3b). Males from CAT mated significantly more often with their own rather than with GYM females. The same was true of SUP males. Significant negative assortative mating, however, was seen between GYM males and SUP females. The deviations from random mated were not predicted on the basis of courtship for the tests using CAT males, but were consistent with the "first female courted" trends seen for SUP and GYM males. In the case of CAT males with GYM females, there was no initial bias toward courting their own females. In summary the outcome of the male choice tests indicate a lack of pre-courtship discrimination by males, but ultimately showed several significant departures from random mating. The departures are consistent with a role of female discrimination or preference in determining courtship outcomes.

The degree to which female strain contributed to these nonrandom mating patterns can be further examined by "female choice" tests (Table 4). These observations, like those in the male choice tests, were broken down into which of the two males was the first to begin courting (Table 4a) the female as well as type of male finally mating (Table 4b). Four significant deviations in first male to court were observed. In three of the four, the first male to begin courtship was from the female's own strain. These deviations are consistent with the slow courtship latencies of LO males when placed with females from other strains (Table 1). As predicted on the basis of courtship latencies, LO males rarely tended to be the first male to court, regardless of female type. There also was a perfect correspondence between the type of first male to court and the male to mate. When females were from CAT, GYM, or the SUP mountains, the significant deviations from random mating were all positive as were the isolation indices. When LO females displayed nonrandom mating, it was always negative assortative mating. Thus in these female choice tests, male propensity, rather than female choice, seemed to have the largest influence on deviations from random mating.

Multiple choice test: Results of multiple-choice tests are presented in Table 5. In only two cases were significant deviations from random mating observed, one involving the CAT x LO pairing and the other in the CAT x SUP tests. Neither case was characterized by a significant isolation index.

Discussion

Drosophila mettleri from different geographic host strains exhibit significant differences in courtship behaviors such as male mating propensity as measured by courtship latency and overall mating speed. Furthermore, these differences in male behavior are influenced, to some degree, by genotypes of females presented to them. While these differences in propensity subsequently influence the outcome of various choice tests, the observed departures from random mating do not suggest strong or consistent sexual isolation among the strains.

Of the four populations used in the present study, flies from Santa Catalina Island exhibit the greatest genetic differentiation from others (Markow et al., 2001; Hurtado et al 2004). It is the Loreto population, however, that exhibits the greatest difference from the others with respect to sexual behavior. Furthermore, there is no evidence that the Santa Catalina Island population exhibits sexual isolation from the other strains.

Data suggest, however, that the outcome of inter-strain pairings is not exclusively dependent upon male or female mating propensity. While it is often difficult to separate sexual vigor from discrimination and preference, the combination of tests used here are able to reveal evidence of mate selection. For example, Loreto males show significantly longer courtship latency when presented with females of strains other than their own. This was associated with longer times until mating as well. An obvious explanation is the reduced ability of these females to stimulate courtship by Loreto males. If this is the

case, it could represent some degree of incipient premating or sexual isolation, but it is clearly not associated with the greater genetic differentiation.

In an earlier study with *D. melanogaster,* Marin (1991) showed that mating choice results at first 50% pairs to mate had a significant value. However, results with mating choice with all potential pairs were different due to *D. melanogaster* vigor. Following Marin (1991), our Chi-Square tests were recalculated to compare early (50%) *versus* all matings. For *D. mettleri*, a multiple choice test between Santa Catalina Island and Guaymas populations was the only combination that showed a discrepancy between early mating results and complete mating results. In this case, the significant result at early mating data was caused by female receptivity. Santa Catalina Island females are essentially receptive to any type of male. On the other hand, Guaymas females are less receptive due to aggressive male behavior in their population. Therefore, when individuals from both populations were placed in a multiple choice arena, Santa Catalina Island females were the first to achieve the mating stage. This explains the significant value at 50% of the potential mates in the arenas ($n=120$; $\chi^2=14.47$; $p<0.01$). However, at the end of the test time, some Guaymas females accepted to mate. Consequently, the statistical result from a complete multiple choice test between Santa Catalina Island and Guaymas populations was not significant ($n=195$; $\chi^2=5.76$; $p>0.05$). It is likely that the early-late difference assumes a greater importance in species like *D. melanogaster* in which copulation last approximately 20 minutes, taking most males out of circulation longer than in other species. In *D. mettleri*, for example, copulation last an average of three and a half minutes and most males are sufficiently vigorous to mate up to four times

in the same period that one mating typically occurs for *D. melanogaster* (Castrezana, *unpublished data*).

Another Sonoran Desert Drosophilidae, *D. mojavensis,* also is found associated with prickly pear cactus on Santa Catalina Island. *Drosophila mojavensis* from Santa Catalina exhibits a degree of genetic differentiation from mainland conspecifics (Ross and Markow, unpublished; Hocutt 2000; Reed and Markow 2004) as observed for *D. mettleri* (Markow *et al*. 2002; Hurtado *et al.*2004). Sexual isolation is observed between *D. mojavensis* from the Baja California peninsula and those from the Sonoran mainland, but not with flies from Santa Catalina (Markow 1991; Hocutt 2000). Taken together, the observations on *D. mettleri* and those for *D. mojavensis* suggest that at early stages of differentiation, no obvious relationship exists between degree of genetic divergence and sexual isolation.

Species of the genus *Drosophila* have provided popular model systems for the study of reproductive isolation (Coyne and Orr 1989, 1997, 1998; Orr 2005) and sexual selection (Boake 2005; Anderson and Kim 2005). Techniques to study deviations from random mating in these studies, however, vary tremendously. Some studies are purely observational, using choice tests and scoring the types of flies mating during a specified period. Choice tests themselves can vary from "male choice" to "female choice" or "multiple choice". Our study clearly shows that these different types of choice tests, when performed with the exact same strains of flies, can give different results and lead to different conclusions. If a literature search and a meta-analysis are performed, combining

results of different kinds of tests might thus lead to erroneous conclusions regarding patterns. Still another type of experiment from which inferences regarding behavioral isolation are made involve choice tests in which two females and a male are left together overnight and evidence of insemination, either by dissection or by presence of progeny, is taken as evidence for or against copulation (Wasserman and Koepfer 1977; Ehrman and Parson 1980; Koepfer and Fenster 1991). Differential sperm storage or utilization could easily masquerade as behavioral isolation. Therefore, it is important to use more than one method to measure reproductive isolation in all *Drosophila* species and to have an understanding of the reproductive biology and ecology of the particular species. In the case of *D. mettleri*, it is clear that a significant amount genetic differentiation doesn't mean that reproductive isolation occurs in allopatric populations. Perhaps some ecological factors such as host shifts, resource distribution, or/and abundance of competitor species play a undetected role in the courtship behavioral differences among *D. mettleri* populations. Additional and more detailed observations of the reproductive biology and physiology of geographic populations of *D. mettleri* and of its closely related species in mainland and Baja, *D. eremophila*, could help to further elucidate the nature of the differences in reproductive biology, the factors underlying the differences, and the potential roles of these differences in incipient speciation.

CHAPTER 3: ECOLOGY, REPRODUCTIVE OUTPUT AND SEXUAL BEHAVIOR DIFFERENCES AMONG ALLOPATRIC *Drosophila mettleri* POPULATIONS

Abstract

The Sonoran Desert endemic *Drosophila mettleri* is a cactophilic soil-breeder species using saguaro (*Carnegiea gigantea*) and cardon (*Pachycereus pringlei*) as a main host in Mexico and Arizona. An isolated population, 300 kilometers NW of the Sonoran Desert limit, was discovered on Santa Catalina Island off the California coast where columnar cacti are not present. This population is genetically isolated from desert populations. Although no consistent behavioral isolation was found among allopatric populations of *D. mettleri,* several other differences in reproductive biology were observed on the island population. Significant differences were found in copulation duration and reproductive output in the insular population where, compared with Sonoran Desert populations, an increase of mating time and a decrease in the offspring per female was observed. On Santa Catalina Island, *D. mettleri* utilizes species of *Opuntia* cacti as its hosts, which contain a chemical and structural composition distinct from the columnar cactus desert hosts. Interestingly, copulation duration and number of offspring produced were inversely correlated. We hypothesize these reproductive differences among allopatric populations are a result of the ecological adaptation process, to *Opuntia*, experienced by *D. mettleri* on Santa Catalina Island.

Introduction

Differences in morphological, physiological and behavioral traits in allopatric populations of the same species are thought to be generated by a combination of mutation, natural selection, and, if there has been a founder event or bottleneck, by genetic drift (Muller 1942; Mayr 1942, 1963; Endler 1986). Under the Biological Species Concept, if genetic differentiation persists, the evolution of prezygotic and/or postzygotic incompatibility may occur between geographically separated populations (Mayr 1963; Noor 1995; Futuyma 1998; Orr 2001), resulting in the formation of distinct species. The nature of traits which, initially have diverged because of ecological pressures, and then subsequently serve to promote incipient speciation remain a major question in evolutionary biology (Wu *et al*. 1995; Schluter 2000).

Behavioral and physiological adaptations to new resources have been proposed as among the first changes to occur when a population shifts into a new adaptative zone (Mayr 1963). In phytophagous insects, host plant chemistry plays an important role on food-related behaviors (Schultz 1988) and the genetics for habitat selection is an intense topic of study (Jaenike and Holt 1991). Some important model system studies of host selection differences in allopatric populations include butterflies (Rausher 1982; Schneider and Roush 1986) and fruit flies (Hoffmann 1985; Jaenike 1986a, 1986b, 1987).

As a general rule, Drosophilidae family species are saprophagous. These flies feed on microorganisms that exist in rotten plant or fungal matter (Fogleman and Danielson 2001). Even though the genetic basis of reproductive isolation between sister

Drosophila species have received considerable attention in the last fifty years (Patterson and Crow 1940; Coyne and Orr 1989, 1997; Orr and Irving 2001), exactly how ecological differentiation in allopatric *Drosophila* populations influences the speciation process has been more or less ignored for the more than 2,500 *Drosophila* species in the world (Kaneshiro 1976; Jaenike 1986a, 1986b, 1987). The close association between Sonoran Desert *Drosophila* and their cacti hosts (Fellows and Heed 1972; Ruiz *et al.*1990) offers an excellent model system in which to address questions about ecological isolation (Heed and Kircher 1965; Zouros and D'Entremont 1980; Heed 1982, 1989; Etges 1992, 1998; Fogleman and Danielson 2001).

Of the four-cactophilic species of *Drosophila* considered endemic to the Sonoran Desert, the last to be described was *D. mettleri* (Heed 1977). Because it is found feeding along side of *D. nigrospiracula* on the same host plant, and is very similar in appearance, it had gone undetected for several decades. In fact, it probably diverged from *D. nigrospiracula* over 30 million years ago (Pitnick *et al.* 1995), and the females oviposit in the soil soaked by necrotic juice as opposed to the necrotic tissue itself, where *D. nigrospiracula* females lay their eggs. While the typical host cacti of *D. mettleri,* the large cardón (*Pachycereus pringlei)* and saguaro (*Carnegiea gigantea*) have a high concentration of alkaloids, these compounds are found in even higher concentrations in the soaked soil where *D. mettleri* develop (Heed 1977, 1978; Fogleman *et al*. 1982).

In the late 1980's an isolated population of *D. mettleri* was found on Santa Catalina Island of the coast of southern California. This new population resides outside

of the Sonoran Desert limits where large columnar cacti are absent (Heed 1989). On this island, *D. mettleri* shifted hosts to prickly pear (*Opuntia)* species. The chemistry and other features of these new host plants are completely different from the columnar cacti, raising questions about the effect of this new host on the ecology as it relates to genetic differentiation and incipient speciation. In fact, genetic studies of *D. mettleri* indicate that individuals in the Santa Catalina Island differ significantly from other *D. mettleri* populations (Markow *et al.* 2002; Hurtado *et al.*2004; Chapter 3). However, p revious experiments concerning the behavior and sexual isolation of *D. mettleri* populations presented in previous chapter did not reveal any significant or consistent patterns of premating isolation among geographically isolated populations of *D. mettleri*.

During the course of rearing the flies for the studies reported in the previous chapter, clear and striking differences were noted in both the reproductive outputs and copulation durations of flies from allopatric populations of *D. mettleri,* with flies from the Santa Catalina Island population having extremely low reproductive outputs. Here, we ask the degree to which populations differ in (1) reproductive output of females and males from each of four geographic regions, (2) the genetic basis of these differences, and (3) if the differences in reproductive output are associated with differences in copulation duration.

Material and methods

Collection and handling of stocks: Collection sites and dates from four geographic populations of *Drosophila mettleri* were as follows: a) Santa Catalina Island, CA (3/97);

b) Superstition Mountains, AZ (3/97); c) Loreto, Baja California, Mexico (11/96); and d) from Guaymas, Sonora, Mexico (11/96) (Figure 1). Each strain was founded from a multiple female collection (n>40) and maintained in large numbers in half-pint bottles on potato cactus medium (Castrezana 1997). For experiments described below, we used the four intrapopulation crosses as well as all sixteen interpopulation crosses and 64 backcrosses.

Measuring reproductive output: Virgin flies were separated by gender under CO_2 and allowed to mature for five days in standard cornmeal vials (10-15/vial) at 24^{o}C. A single female and a single male were placed in a shell vial and observed for 20 minutes or until copulation occurred. Mated females were then placed individually in a half-pint bottle prepared with 20 ml. of potato cactus medium and transferred daily into a new bottle during four days. Females were then discarded. Bottles were examined 10 days after copulation to determine if larvae were present. For each combination, a minimum of three females was used. Emerging offspring were counted by gender every day from 15 to 41days after copulation occurred.

Measuring copulation duration: Virgin flies were separated and maintained as above. Mature pairs of virgin flies were placed in vials and observed until copulation occurred or 15 minutes had elapsed. Copulation duration was recorded in seconds. For each cross, a minimum of 24 successful mating pairs was observed.

Statistical analysis: Reproductive output and copulation duration were subjected to factorial ANOVA with male and female strain as the two factors. The analysis was conducted using JMP software version 4.0.4 (SAS Institute Co., 2001).

Results

Reproductive output: The proportion of matings successfully producing offspring, the total offspring, and the average offspring per female for single matings of pairs from each population are presented in Table 6a. Average productivity varied significantly among the populations, with those from mainland, Superstition and Guaymas, producing three to four times as many offspring as those from Santa Catalina Island. The multiple range test placed populations in overlapping subsets, with the exception of the Santa Catalina Island strain. Interestingly, only about half of the copulations observed for pairs from the Guaymas strain produced progeny, although this strain, when they did produce progeny, produced a large number of them.

In order to investigate the influence of male and female strain on reproductive output, a series of interpopulation matings was conducted and progeny counted. The results, summarized in Table 6b, reveal two things. First, there is no consistent evidence of incompatibility between the strains, either in the form of reduced number of productive matings or the average number of progeny per productive mating. The exception was in matings between Superstition females and Santa Catalina Island males, where the proportion of matings yielded low progeny (33%). Second, the low number of progeny produced by flies from the Santa Catalina Island strain, relative to the others, is a function

of both sexes. Regardless of the origin of the other strain, on average, males and females from Santa Catalina Island produce (significantly) half the progeny observed for all other interpopulation matings (Table 7).

The genetic basis of the low productivity of Santa Catalina Island flies was investigated by mating F1 progeny from crosses between the Santa Catalina Island and Guaymas strains, as these two exhibit the greatest degree of difference for this trait, and mating them to either parental strain (Table 8). The strain or genotype of the female is always listed first. Reciprocal crosses created F1 progeny used in these backcrosses in order to reveal evidence of sex linkage or maternal effects. If a gene or genes on the X chromosome of males has a significant influence on their reproductive output, males whose X chromosome came from the Guaymas population should produce more offspring than those whose X-chromosome came from the Santa Catalina Island population. Because females from the two populations exhibit significant differences in reproductive output, it is necessary to compare the effect of the different X-chromosomes in matings to the same type of female. From Table 8, it is clear that the low productivity of Santa Catalina Island females obscures our ability to detect differences between males whose X-chromosome came from Santa Catalina Island versus Guaymas populations. When the comparison is made, however, using females from Guaymas, the impact of the Santa Catalina Island X-chromosome on the productivity of the mating is clear. Almost half as many progeny are produced when sires have the Santa Catalina Island X-chromosome ($df=1$, $F=29.629$, $P<0.001$), suggesting that a gene or genes of major effect on male productivity are sex-linked.

Copulation duration: In order to ask if the differences among geographic strains can be attributed to differences in the duration of mating, copulation duration for all four strains was recorded (Table 9). Significant differences in copulation duration among populations were observed. Contrary to what is expected if copulation duration is positively associated with the number of offspring produced, the Santa Catalina Island population had the longest copulation time with more than eight minutes, 30% longer than that of the time used by mainland populations (Table 9a). On the other hand, no statistical differences between mainland populations were found. Both populations spent approximately six minutes in copula.

When copulation durations of interpopulation pairings were measured, significant difference among females, males and their interaction were observed (Table 10). Regardless of the origin of mating partner, *D. mettleri* individuals from Santa Catalina Island copulate significantly longer than individuals from other *D. mettleri* populations. Furthermore, within Santa Catalina Island individuals, despite the source of copulation partner, Santa Catalina Island males used an average of 40 seconds more time to copulate than Santa Catalina Island females (Figure 4). Finally, when average reproductive output and copulation duration were compared, an inverse relationship was observed: as mating time increased, reproductive output decreased (Figure 5).

Discussion

The number of offspring produced by a single mating in *D. mettleri* has significant variability depending on the geographic strain. Flies from Santa Catalina

Island produced only about ¼ of the offspring produced by mainland populations. The low output is a function of both sexes from this *D. mettleri* strain: males or females from other strains, when mated to Santa Catalina Island flies, produce fewer progeny. The fact that both sexes of the Santa Catalina Island strain exhibit low productivity, compared to the other strains, suggests that male and female productivity have coevolved.

Although *Drosophila* literature contains abundant studies of species with some degree of sexual isolation among their allopatric populations (Stalker 1942; Malagolowkin-Cohen *et al.* 1965: Zouros and D'Entremont 1980; Markow *et al.* 1981; Coyne and Orr 1989, 1997), few studies examine in detail the reproductive output and mating time differences on allopatric populations as initial outcomes of an incipient speciation process. An example occurs with species in the *D. ananassae* subgroup where reproductive output differences in allopatric populations were associated with copulation duration (Singh 1996; Singh and Singh 2001). However, in the case of *D. mettleri* we observed a negative connection between copulation duration and reproductive output where populations with the higher mating time produced less offspring. Therefore, reproductive output and mating time probably are traits under different selective pressures.

On the other hand, we also observed significant variation in the copulation time among allopatric populations of *D. mettleri*. Copulation duration varies widely in Drosophilidae family (Spieth 1952, Grant 1983; Markow 1996). However, the biological significance of these differences has been elusive, as they do not appear to correlate with

other characters. Few exceptions appear in members of the *D. ananassae* subgroup (Singh 1996; Singh and Singh 2001). In *D. mettleri* an increase of mating time is a function of both sexes from Santa Catalina Island individuals. However, Santa Catalina Island males used considerable more time to copulate than their own females. A similar situation occurs in *D. melanogaster* where copulation duration was demonstrated to be a heritable male trait (MacBean and Parson 1967; Gromko *et al.* 1991). Unfortunately, most of the research concerning copulation time in *Drosophila* deals only with interspecific variability but not with intraspecific variation, and papers are mainly limited to the *D. melanogaster* subgroup (Robertson 1983; Coyne *et al.* 1991; Coyne 1992, 1993).

One can only speculate about the potential ecological factors that may have driven the differences in reproductive biology among the strains. *Drosophila mettleri* is considered to be a soil breeder using the soil soaked with necrotic juice from decaying columnar cacti either saguaro or cardón in Arizona-Sonora and Sonora Baja respectively (Heed 1977, Heed 1978). It is important to note that necrotic material from columnar cacti contains a high concentration of alkaloids, such as gigantine and carnegine, lethal to non-Sonoran Desert *Drosophila* (Kircher *et al.* 1967; Kircher 1982). Furthermore, the high evaporation rate for necrotic pools forces *D. mettleri* larvae in peninsular and mainland populations to tolerate allochemical concentrations up to 27 times greater than other Sonoran Desert *Drosophila* (Fogleman and Williams 1987; Meyer and Fogleman 1987; Fogleman and Danielson 2001). In contrast, *D. mettleri* from the Santa Catalina Island strain differs from the others in that columnar cacti do not grow on Santa Catalina

Island. The only cacti available for *D. mettleri* on the island are plants from the genus *Opuntia* which do not contain the toxic allochemical deterrents normally present on columnar cacti (Heed 1989; Danielson *et al.*1997). In addition, these plants in Santa Catalina Island are hosts for several highly fecund species such as *D. mojavensis*, *D. hamatofila* and *D. wheeleri*. It is not clear if *D. mettleri* larvae on Santa Catalina Island are using the soil soaked by necrotic *Opuntia* (Heed, pers. comm.; Ross, pers. comm.), but in recent collections, *D. mettleri* was reared from necrotic tissue along with *D. wheeleri* (Castrezana unpublished data).

Although nutrients such as nitrogen and phosphorous play an important role influencing resource and reproductive ecology of *Drosophila* (Markow, *et al.* 1999), prickly pears may provide a radically different habitat. In the case of *D. mettleri* it is possible that differences other than nutrients could play an important role in the adaptation to the novel host. First, necroses in columnar cacti last longer than *Opuntia*. A necrotic phase in a giant cardon cactus can persist two years creating a constant breeding niche for D. *mettleri* (Castrezana, unpublished data). On the other hand, single necrotic *Opuntia* pads in shade endure 20-35 days. However, in the presence of *Drosophila,* prickly pear necroses last 30-50% less than non colonized pads (Castrezana, unpublished data). Second, toxic alkaloids in columnar cacti prevent interspecific competition and larvae predation. *Drosophila mettleri* is the only species in the Sonoran Desert able to support a concentration higher than normal for lethal cacti chemicals such as alkaloids, sterol-diols, medium chain fatty acids, and triterpene glycosides (Frank and Fogleman 1992). In necrotic pool samples from saguaro and cardon cacti collected in Arizona,

Sonora, and Baja, all but one of 1,254 individuals reared were *D. mettleri* (Castrezana, unpublished data). On the other hand, *D. mettleri* was not present in soil samples beneath *Opuntia* spp. necroses. *Drosophila mettleri* individuals from Santa Catalina Island compete for larvae resources with *D. hamatofila* and *D. wheeleri*. Only 15% of the *Drosophila* reared from necrotic *Opuntia littoralis* pads were *D. mettleri* (Castrezana, unpublished data). In contrast, in Arizona, *Drosophila* reared from necrotic *Opuntia* spp. were *D. aldrichi, D. longicornis, D. hamatofila, D. arizonae* and *D. hydei* (Castrezana, unpublished data). On the other hand, larvae predators (Dermaptera, Coleoptera and Hymenoptera) are common in *Opuntia* necroses (Castrezana, unpublished data). These observations allow us to speculate that decreases in reproductive output in the *D. mettleri* Santa Catalina Island population could be a response to the size reduction and chemical composition change in the novel host, which create a high interspecific competition for larval resources.

Without the genetic tools available in *D. mettleri* that are available in *D. melanogaster,* we can only show that for females, reproductive output is likely to be a quantitatively inherited trait. F1 females between Guaymas and Santa Catalina Island are intermediate in the reproductive output. On the other hand, because *Drosophila* males are the heterogametic sex and therefore hemizygous for genes on their X chromosomes, we can show that male productivity in these two strains is strongly influenced by a gene or genes on the X chromosome.

It is clear that we need to understand the genetic basis of reproductive isolation in order to better understand the speciation process. However, we cannot limit our knowledge of the speciation process to species with some already significant degree of premating or postzygotic isolation among their populations. The process of new species formation involves time to accumulate genetic changes for individuals to adapt to novel ecological niches even before any reproductive barrier are former. *Drosophila* species with allopatric populations can provide clues to understand some of the broad ways in which the speciation process may emerge previous to the reproductive, premating and postzygotic, isolation barriers. *Drosophila mettleri,* with behavioral and physiological differences between allopatric populations, provide an opportunity to investigate subtle differences in reproductive biology that may represent extremely early incompatibilities in the speciation process. Further detailed experiments among allopatric populations of *D. mettleri* on factors such as sperm length, oviposition site preference, larva performance, development time and success may provide clues regarding the course of incipient speciation.

CHAPTER 4: POSTMATING ISOLATION AMONG GEOGRAPHIC POPULATIONS OF *Drosophila mettleri*

Abstract

Nei's genetic distance of 0.5 is associated with emergence of both prezygotic and postzygotic reproductive isolation between sister *Drosophila* species. However, in allopatric populations within a species, the amount of genetic differentiation observed before incipient reproductive isolation appears is undefined. The cactophilic Sonoran Desert endemic *Drosophila mettleri* is a soil breeder species associated with saguaro and cardón cacti in mainland and Baja California respectively. An additional population recently was found on Santa Catalina Island off the Coast of California 300 km away from the Sonoran Desert, where it had shifted host to *Opuntia* spp. We used alloenzyme electrophoresis and mitochondrial cytochrome oxidase subunit I gene (*mtCOI*) to determine population genetic differentiation among all of the regional populations of *D. mettleri*. Then, we compared results from both methods. Finally, for each population and its hybrids, we investigated development time and male sterility to identify signs of incipient postzygotic isolation. We found that *D. mettleri* from Santa Catalina Island was significantly genetically differentiated from all other populations in the study. In addition, although a complete postzygotic isolation was not detected among *D. mettleri* populations, we observed sufficiently significant F_1 male sterility in crosses involving the Santa Catalina Island population to consider it indicative of early postzygotic isolation.

Introduction

The evolution of a complete lack of interbreeding between individuals from diverged populations remains a fundamental problem to our understanding of the speciation process. Ecological hypothesis proposes than reproductive isolation among populations in different environments is caused by the accumulation of genetic differences resulting from divergent natural selection (Mayr 1942, 1963; Dobzhansky 1951).

Nevertheless, we remain unaware about the type and magnitude of genetic changes that occur during species formation. Perhaps the reason is that few studies focus on the genetic changes that accompany the evolution of reproductive isolation (Via and Hawthorne 1998). Important exceptions are the comprehensive studies on *Drosophila* postmating isolation, both at interspecific (Coyne 1984; Coyne and Orr 1989, 1997; Orr 1989; Price *et al.* 2001; Sun *et al.* 2004) and intraspecific levels (Hollocher *et al.* 1997; Alipaz *et al.* 2005). Postmating isolation may take the form of hybrid inviability or sterility. When only one sex is affected, typically it is the heterogametic sex (Haldane 1922), which is one reason why hybrid males sterility is the trait typically scored in studies of postmating isolation. Nevertheless, it is useful to concentrate on species whose populations have undergone recent vicariant events in order to examine the ecological forces and genetic changes of the speciation process at early stages.

An exceptional geographical opportunity to investigate the process of how new species arise in terrestrial organisms is the Sonoran Desert, which was divided by the

Gulf of California approximately three to six million years ago (Gastil *et al.* 1983). This vicariant event created a 1,100 km long and 120 km maximum wide separation between peninsular and continental desert communities. Over the last 40 years four Sonoran Desert endemic *Drosophila* and their columnar cacti hosts offered exceptional model systems to address questions about host plants associations (Fellows and Heed 1972; Fogleman *et al*. 1981; Ruiz and Heed 1988; Fogleman and Danielson 2001), population genetics (Zouros 1973; Pfeiler and Markow 20001a) and speciation (Zouros and D'Entremont 1980; Markow 1981; Markow *et al.*1983; Etges 1992).

Sonoran Desert *Drosophilidae* are known for their independent adaptation to chemical compounds in large columnar cacti, which are toxic to all non-Sonoran *Drosophila* species (Kircher *et al.* 1967; Fogleman *et al.* 1981; Kircher 1982; Frank and Fogleman 1992). The most recently described *Drosophila* endemic to the Sonoran Desert is *D. mettleri*, a species in the *D. eremophila* complex of the *D. repleta* group (Heed 1977). Adults of cactophilic *D. mettleri* are extremely similar to *D. nigrospiracula* and both species feed on the same host cacti such as cardón (*Pachycereus pringlei)* in Baja California and south of Sonora, and saguaro (*Carnegiea gigantea*) in Sonora-Arizona (Heed 1982). However, *D. nigrospiracula* uses necrotic tissue for larval niche whereas *D. mettleri* uses the soil soaked by necrotic juice from its large columnar hosts (Heed 1978; Fogleman and Williams 1987). The high evaporation rate in the Sonoran Desert increases the alkaloid concentration in soil soaked by necrotic juice more than 27 times the alkaloid concentration present in necrotic tissue (Fogleman *et al.*1982). This alkaloid concentration is lethal not only for *D. nigrospiracula* larvae but also for those *Drosophila*

larval predators present in the necrotic tissue of the large columnar cacti (Castrezana, unpublished data).

On the other hand, an isolated population of *D. mettleri* outside of the Sonoran Desert limits was found on Santa Catalina Island in the Southern Coast of California where columnar cacti are not present. This *D. mettleri* population shifted its hosts to *Opuntia demissa, O. oricola* and *O. littoralis* (Heed 1989; C. Ross, pers. Comm.). However, decayed *Opuntia* cannot produce enough fermented material to soak the soil for several weeks (Castrezana, unpublished data), and they do not contain toxic allolechemical deterrents normally present in Sonoran Desert columnar cacti (Fogleman *et al.* 1982; Danielson *et al.* 1997; Fogleman and Danielson 2001). Therefore, chemical, morphological, and availability differences among geographic hosts of *D. mettleri* instigate questions about potential roles of these factors in incipient speciation.

A previous study found no notable genetic differentiation between populations of *D. mettleri* across great distances in mainland Sonoran Desert (Pfeiler and Markow 2001a). In addition, our other studies (Chapter 1) did not find evidence for strong premating isolation among allopatric populations across the geographical range of *D. mettleri.* However, we discovered significant differences in mating propensity, sexual behavior, copulation duration, and reproductive output between *D. mettleri* continental populations and the Santa Catalina Island strain, demonstrating that differentiation has occurred in some traits. Two questions remain unanswered, however. The first is the degree to which the various geographic populations of *D. mettleri* are genetically

differentiated from each other and the second is whether there is any evidence of postmating isolation.

Here, we center the attention of our research on the population genetics and postzygotic isolation in all the geographical distribution of *D. mettleri*. The following questions were asked: (i) what is the degree of genetic differentiation among populations of *D. mettleri* from different parts of its range? (ii) Is there any evidence of postmating isolation among *D. mettleri* populations and, if so, is it associated with the degree of genetic differentiation? We utilized nuclear and mitochondrial markers to examine genetic differentiation. To examine postmating isolation, we examined three characters: male sterility, development time, and sex ratio in F1 hybrids between the geographic populations.

Material and methods

Collection and handling of stocks: Collection sites and dates for field caught individuals in fourteen localities used to determinate population genetics and postzygotic isolation are shown in Figure 6. Although strains used in both alloenzyme electrophoresis and *mtDNA* sequence studies were not identical, both types of genetic studies showed the differentiation patterns among populations (Markow et al. 2002; Hurtado *et al.* 2004).

Allozyme electrophoresis: 275 *Drosophila mettleri* adults from eight localities were collected from natural necrotic cacti or artificial necrotic cactus baits. Adults collected in saguaro rotten tissue (*C. gigantea*) were obtained in Tucson, AZ (02/2000), and Organ Pipe Cactus National Monument Park, AZ (09/2000). Adults collected in cardón rotten

tissue (*P. pringlei*) were obtained in Guaymas, Sonora, Mexico (11/1998), Bahía Concepción and Ensenada de los Muertos, Baja California South, Mexico (01/2001). Adults collected in senita rotten tissue (*Lophocereus schottii*) were obtained from Cataviña and Highway 1, km249, both in the state of Baja California, Mexico (01/2001). Adults were also collected in prickly-pear *Opuntia littoralis*-banana bait in Santa Catalina Island, CA (04/2001). The number of flies analyzed in each locality ranged from 30 to 70 individuals (Markow *et al.* 2002).

*mt*DNA *sequences*: 117 *D. mettleri* adults from eight populations were collected from natural necrotic cacti or from artificial baits. Adults collected from rotting saguaro tissue came from Tucson, AZ (03/2002) and Sierra Ancha, AZ (02/2003). Adults collected from cardón rotten tissue were obtained in the follow places: Lago Chapala Baja California North (01/2001); Bahía Concepción El Cien, and Punta Conejo, Baja California South (01/2001); and Guaymas, Sonora, Mexico (03/2002). In Santa Catalina Island, CA (10/2002) adults were collected from banana bait. The number of flies analyzed in each locality ranged from 5 to 20 individuals (Hurtado *et al*. 2004).

Postzygotic isolation: For development time and male sterility, four geographical strains of *D. mettleri* were founded from multifemale collections (n>40) from natural necrotic cacti. Collection sites and dates were as follows: Strain founders collected in necrotic saguaro cacti from Superstition Mountains, AZ (03/1997). Strain founders collected in necrotic cardón cacti were obtained in Loreto, Baja California South, Mexico (11/1996) and Guaymas, Sonora, Mexico (11/1996). Strain founders collected in necrotic

prickly-pear *O. littoralis* were from Santa Catalina Island, CA (03/1997). These populations were maintained in large numbers in half-pint bottles on potato-cactus medium (Castrezana 1997). Founder individuals used in postzygotic isolation research were different from those individuals used in population genetic studies. However, similar geographical *D. mettleri* populations were cover in both experiments.

Population genetics methods and analyses: Genetic differentiation among geographic populations of *D. mettleri* was examined using both allozyme electrophoresis (Markow et al. 2002) and by mtDNA sequences (Hurtado et al. 2004).

Allozyme electrophoresis: adults were separated by gender and homogenized individually in 25µl of grinding buffer (Cleland et al. 1996). Homogenates were centrifuged for 5 min at 10,000 g and the supernatants analyzed by electrophoresis either on 12.5% starch gels (Starch Art Corp., Smithville, TX) or Titan III cellulose acetate plates (Helena Laboratories, Beaumont, TX). Starch gel electrophoresis was carried out at 4^{o}C in a buffer system of 40 mM citrate adjusted to pH 6.0 with N-(3-aminopropyl) morpholine (dilution in gel 1:20). Following electrophoresis, gel slices were stained for enzyme activity using Murphy et al. (1990) recipes. Cellulose acetate electrophoresis was performed for 2 min at 22^{o}C/200 V and using Tris-glycine buffer (pH 8.0); enzyme staining was according recipes in Herbert and Burton (1989) with minor modifications.

The eight enzymes analyzed for *D. mettleri* were as follows (with abbreviations and EC numbers): phosphoglucomutase (PGM, 5.4.2.2); alcohol dehydrogenase (ADH,

1.1.1.1); malate dehydrogenase (MDH, 1.1.1.37); glycerol3-phosphate dehydrogenase (NAD^+)(GPDH, 1.1.1.8); cytosol nonspecific dipeptidase (PEP-A, 3.4.13.18, glycylleucine substrate); tripeptide aminopeptidase (PEP-B, 3.4.11.4 leucyglycylglycine substrate); arginine kinase (ARGK, 2.7.3.3); and carboxylesterase (EST, 3.1.1.1, α-napthylacetate substrate). The loci coding for these enzymes are abbreviated with italics. There was not evidence for sex-linkage, or sex specific suppression of enzyme activity (Pfeiler and Markow 2001), for any of the loci examined. Therefore, allele frequency data for both sexes were combined for population analysis. (Markow *et al.*2002).

Estimates of genetic variation and Wright's *F*-statistics were performed with BIOSYS-1 (Swofford and Selander 1989). The calculation of significance of pairwise comparisons of F_{ST} was performed with Arlequin version 2.000 (Schneider *et al*. 2000) using 1000 permutations of the data matrix.

*mt*DNA *studies*: Entire flies were ground for DNA extraction. DNEasy kit protocol was used for total DNA extraction in each individual (Qiagen, Inc. Valencia, CA). A 710-base-pair fragment of the *mtCOI* gene was amplified from all flies analyzed. DNA primers and polymerase chain reaction (PCR) conditions are described in Folmer *et al.* (1994). The PCR products were sequenced in both directions using an ABI3700 analyzer. Sequences were proofread and aligned with SEQUENCHER vs. 4.1 (Gene Codes Corp.). Assembled sequences were truncated to a fragment that contained only 663 readable nucleotides. Then, *mtCOI* DNA sequences were translated to amino acid sequences. No pseudogenes or another DNA fragment were found.

Statistics calculated were *mtCOI* haplotype diversity (equation 8.6 in Nei 1987); mean number of pairwise differences (Tajima 1983); and nucleotide diversity (equation 10.6 in Nei 1987) as implement implemented in ARLEQUIN 2000 (Schneider *et al.* 2000). PAUP vs. 4.0b8 was used to calculate maximum parsimony and neighbour-joining analyses (Swofford 1998). Maximum parsimony analyses were performed by heuristic searches with 50 stepwise random additions and three bisection-reconnection (TBR) branch swapping. Bootstrap support values were calculated from 50% majority-rule consensus tree based on 1000 bootstrap replicates. Statistical parsimony haplotype networks were constructed using the TCS program (Clement *et al.* 2000). They may provide better representation of gene genealogies at the population level (Templeton *et al.* 1992).

Values of genetic differentiation between populations (F_{ST}), the corresponding number of migrating individuals (*Nm*) were calculated following Hudson *et al.* (1992). For haploid data, such as *mtDNA*, *Nm* is calculated as $[(1/F_{ST})-1]/2$. Analysis of molecular variance (AMOVA, Excoffier *et al.* 1992) as implement of ARLEQUIN 2000 was also conducted. AMOVA estimates the amount of genetic variation attributable to genetic differentiation among group (Φ_{CT}), among localities within groups (Φ_{SC}), and among localities relative to the total sample (Φ_{ST}).

Postmating isolation methods and analyses

Measuring development time. Virgin flies were separated by gender under CO_2 and allowed to mature for five days in standard cornmeal vials (10-15/vial) at 24°C. A single

female and a single male were placed in a shell vial and observed for 20 minutes or until copulation occurred. Mated females then were placed individually in a half-pint bottle prepared with potato cactus medium (Castrezana 1997), and transferred daily into fresh food bottles daily for four days. Then, females were discarded. Bottles were examined 10 days after copulation to determine if eggs/larvae were present. Emerging offspring were counted by gender every day from day 15 to 41 after copulation occurred. Identical methodology was employed for control and F1 lines. Development time was subject to factorial ANOVA with male and female strain as the two factors. The analysis was conducted using JMP software version 4.0.4 (SAS Institute Co., 2001).

Measuring male sterility: From the experiment above, three virgin male offspring from each culture of the four *D. mettleri* populations and their reciprocal interpopulation crosses were separated under CO_2. *Drosophila mettleri* males have an aggressive sexual behavior. Therefore, virgin males were allowed to mature individually in standard cornmeal vials for ten days. Male reproductive tracts were dissected under a dissecting microscope and placed on a slide with a saline solution. Then, reproductive tracts were squashed with a cover slip. Slides were carefully checked for sperm motility. Data are presented as percentage of males without motile sperm (sterility).

Results

Population Genetics Results

Allozyme electrophoresis: Alleles frequencies at each locus obtained for *D. mettleri* were used to calculate average genetic variability in allopatric populations (Table 11).

Highway 1 km249 population had a significant heterozygote excess for the *Adh* fast/slow polymorphism (Fixation index=0.403; D=0.385). Mean observed heterozygosity (H_o) averaged across all *D. mettleri* populations was 0.100±0.006.

F_{ST} values for eight populations of *D. mettleri* are shown in Table 12. A mean F_{ST} of 0.194 was obtained for all *Drosophila mettleri* populations. Clear geographical separations occurred in *D. mettleri* populations. When pairwise comparisons of overall F_{ST} for three *D. mettleri* mainland populations were compared each to the four peninsular populations, significant values among seven of 12 possible pairwise comparisons were found (Table 13a). Additionally, all pairwise comparisons of the Santa Catalina Island population with all other populations showed highly significant F_{ST} values. Finally, two from six pairwise comparisons within Baja California populations were significant. Both comparisons involve the northeast Baja California population in the middle of the mountains.

Allele frequencies at two loci, *Pgm* and *Adh*, highly influenced the F_{ST} values. The predominant allele *Pgm* (*155) had a frequency of 0.750 in the Santa Catalina Island population. However, this allele was present in only one individual from 245 individuals analyzed from all other populations. In the case of locus *Adh*, *D. mettleri* has a fast (*-100) and slow (*-81) allele. These alleles had similar frequencies in peninsular and continental *D. mettleri* populations. However, in Santa Catalina Island population the slow *Adh* allele had a high frequency of 0.817

*mt*DNA: A total of 117 individuals were used to determine an estimate of genetic variation of π_1=1.37 in *D. mettleri* (π_1=mean number of pairwise differences). Twenty four different haplotypes were discovered from all *D. mettleri* individuals sequenced. These data were incorporated to GenBank with accession numbers AY533789 to AY533812. The most common haplotype observed was present in 64% of the continental individuals and 57% of peninsular individuals. Identical topology of the 24 different haplotypes was obtained with statistical parsimony, maximum parsimony, and neighbour joining. Figure 7 shows the haplotype network. From these topologies, Santa Catalina Island had three unique haplotypes. These Santa Catalina Island haplotypes showed a clear separation from continental/peninsular haplotypes. However, no evidence for separation between continental and peninsular populations was observed from these topologies.

On the other hand, *D. mettleri* from Santa Catalina Island was significantly genetic different from all the other populations examined (Table 13b). In addition, two south peninsular localities, Bahía Concepción and Punta Conejo, were significantly different versus other localities. No significant genetic differentiation between continental and peninsular ranges (Φ_{CT}=0.000, Φ_{SC}=0.055, Φ_{ST}=0.043) (Hurtado *et al.*2004).

Postmating Isolation Results

Development time: Development time in four intrapopulation (control) and twelve interpopulation crosses of *D. mettleri* is shown in Figure 8. We expected to see faster development time in *D. mettleri* populations from Loreto and Santa Catalina Island since

these populations use a host cactus with significantly smaller dimensions than the flies from Sonoran. The 19 days of development time for *D. mettleri* individuals from Santa Catalina Island and Baja California populations were significantly shorter than 21 days of development time obtained in continental populations. In fact, hybrid individuals of *D. mettleri* were significantly equivalent to their Santa Catalina Island parents rather than continental parents ($df=15$; $F=231.4514$; $p<0.001$).

Sex ratio: Sex ratio in four intrapopulation and twelve interpopulation crosses of *D. mettleri* is shown in Figure 9. In the case of a postmating isolation, we expected to observe a significant bias in the sex ratio especially between Santa Catalina Island and continental populations. Our results showed a female bias in all crosses. However, none of the differences in sex ratio were found to be statistically significant ($df=15$; $F=0.955$; $p=0.5050$).

Male sterility: Haldane's rule indicates that sterility in hybrid heterogametic individuals is one of the earliest manifestations of postzygotic isolation between species. Therefore, if *D. mettleri* populations are undergoing incipient speciaton, we expect to see some degree of male sterility in hybrid individuals from crosses between populations from Santa Catalina Island with those from Guaymas, the most remote continental population. While we in fact observed 21% sterility in hybrid males from the cross between Guaymas females and Santa Catalina Island males, a remarkable 50% male sterility was found in hybrids from Superstition females crossed with Santa Catalina Island males (Figure 10).

Discussion

We were interested in obtaining the degree of genetic differentiation among populations of *D. mettleri* using two molecular methods and checking if both techniques showed similar patterns. Additionally, we explored evidence of postzygotic isolation and its potential association with genetic differentiation among *D. mettleri* populations. Alloenzyme electrophoresis and mtDNA analyses revealed that *D. mettleri* from Santa Catalina Island is significantly genetically differentiated from all other localities compared. Furthermore, both molecular studies indicated that *D. mettleri* exhibit significant genetic differentiation within Baja California populations Perhaps the unique geological history of the Baja California peninsula not only influences the phylogeographic population structure of vertebrate species (Riddle *et al.* 2000) but may also influence some invertebrates too.

Another Sonoran Desert species, *D. mojavensis*, presents a similar geographic distribution to *D. mettleri,* and it shifts hosts from columnar cacti to *Opuntia* spp. in Santa Catalina Island (Heed 1989). In addition, the *D. mojavensis* population from Santa Catalina Island is significantly genetically differentiated from Baja California and mainland populations (Hocutt 2000). Although *D. mettleri* exhibits some degree of genetic differentiation among Baja California and mainland populations, *D. mojavensis* displays greater genetic isolation between peninsular and continental populations (Zouros 1973; Hocutt 2000). However, *D. mojavensis* did not exhibit genetic differentiation within Baja California populations (Etges 1992). Perhaps the lack of genetic differentiation in *D. mojavensis* is due to the unique host in Baja California. On the other

hand, *D. mettleri* is capable to surviving high concentration of toxic chemicals present in all columnar cacti (Frank and Fogleman 1992). This ability of *D. mettleri* may support the existence local populations and influence the gene flow among Baja California populations. Markow *et al.* (2002) showed that *D. mettleri* and *D. nigrospiracula* have a lack of significant genetic differentiation across the Gulf of California. *Drosophila mettleri* and *D. nigrospiracula* are extremely similar in size (Fogleman *et al.* 1981), they share the same host plants in Baja California and the mainland (Heed 1977), and both can be occasionally collected feeding in a wide variety of cacti (Fellows and Heed 1972). Nevertheless, dispersal data are available only for *D. nigrospiracula*, which is classified as a strong disperser (Markow and Castrezana 2000). Genetic differentiation results along reproductive differences observed within Baja California populations may not support the idea of *D. mettleri* to be as strong a disperser as *D. nigrospiracula*. On the other hand, genetic variability in *D. mettleri* from Santa Catalina Island may suggest multiple colonization events rather than a bottleneck associated with the founding of this island population (Markow *et al.* 2002).

The host shift from columnar cacti to *Opuntia* spp. in *D. mettleri* from Santa Catalina Island may be a factor underlying the significant genetic isolation observed between this and other *D. mettleri* populations. Host shifts are suggested to be the initial step in the speciation process in phytophagous insects and animal parasites (Jaenike 1978; Bernays and Chapman 1994). In fact, it is proposed that variation in gene(s) controlling acceptance/rejection of the plants chemicals may underlie the first behavior involved in host shifts (Singer 1971; Jaenike 1978; Courtney *et al.* 1989). On Santa Catalina Island,

D. mettleri was reared directly from necrotic pads of *Opuntia littoralis* (Castrezana, unpublished data). Conversely, *D. mettleri* larvae in Sonoran Desert populations are reported to use only the soil soaked by necrotic juice from saguaro or cardón cacti (Fogleman *et al.* 1981). The extraordinary resistance of *D. mettleri* to a wide variety of toxic deterrents, including those chemical concentrations which are lethal to the Sonoran Desert *Drosophila* (Frank and Fogleman 1992; Fogleman and Danielson 2001), was almost certainly the genetic basis to colonize the novel host in Santa Catalina Island. In fact, *D. mettleri* larvae from Santa Catalina Island retained the capability to survive in lethal Sonoran Desert alkaloids in addition to the novel *Opuntia* host adaptation (Castrezana, unpublished data).

In our second question, we examined the relationship among postzygotic isolation and genetic distance. If *D. mettleri* undergoes postmating isolation, we expected to observe some degree of deviation from the normal character expression in hybrid individuals when an artificial encounter occurred between Santa Catalina Island and continental individuals. In previous studies of other traits presented in chapter two, such as copulation duration and reproductive output, *D. mettleri* hybrids were equivalent to their Santa Catalina Island parents, a result more consistent with hybrid vigor rather than postmating isolation. In this chapter however, an early form of postzygotic isolation was found in *D. mettleri* since an important percentage of F_1 male sterility occurred mainly among population crosses involving individuals from Santa Catalina Island. On the other hand, we found a significant reduction in the development time for Santa Catalina Island and Baja California populations. However, development time was not a important

character to detect postmating isolation since our results had similar patterns that observed in previous studies: hybrid expression were equivalent to their Santa Catalina Island parents. Therefore, genetic distance differences observed in *D. mettleri* among Santa Catalina Island with other populations probably reflected the incipient stage of postzygotic isolation we observed. Coyne and Orr (1989; 1997) reviewed intensively premating and postmating isolation stages of speciation, both interspecific and intraspecific, in the *Drosophila* literature. One of their conclusions widely corroborated Haldene's rule that the heterogametic sex is the most affected during hybridization and therefore, it is considered the initial stage for of postzygotic isolation. Furthermore, genetic studies on postmating isolation in *Drosophila* explained the genetic basis of F_1 male sterility, which is more complex than the genetic basis of hybrid inviability, which is the third step suggested in the speciation process (Hutter 2002; Sun *et al.* 2004). Therefore, we can suggest than *D. mettleri* individuals from Santa Catalina Island are genetically different in multiple ways from to other D. *mettleri* populations, not only for the incipient postzygotic isolation, but also for all physiological and ethological genetic changes implied in a host shift.

In conclusion, detailed postzygotic isolation, ecology, behavior and population genetic studies in species such as *D. eremophila, D. spenceri, D. arizonae* and *D. pachea* with populations in both sides of the Gulf of California may increase our knowledge about ecological and genetic circumstances than facilitate speciation at early stages. Questions such as what happened before male sterility arose between populations and how much genetic changes must be attained before we can consider two geographically

isolated organisms to be on the initial steps of the speciation may help to understand early genetic patterns of an incipient reproductive speciation. Finally, *D. mettleri* as a model of speciation still has several questions about female preference, larval performance, climatic limitations, interspecific competition and chemical adaptations among populations that may help to understand how *D. mettleri* evolved, how it settled in the Sonoran Desert, and incipient speciation began.

CHAPTER 5: SUMMARY

In Chapter Two, we tested for evidence of sexual isolation among allopatric populations of *D. mettleri* using several mate choice tests. Contrary to what was expected, *D. mettleri* from the Loreto population exhibited the greatest difference from the others with respect to sexual behavior. Males showed significantly longer courtship latencies and consequently mating times. One apparent explanation is the decreased ability of Loreto females to stimulate courtship by Loreto males. If this is the case, it could contribute to incipient premating or sexual isolation at some point, isolation which is obviously not associated with the genetic differentiation observed. However, in general we concluded that male behavior differences in *D. mettleri* populations are influenced by the genotypes of the females presented to them. While these differences in mating propensity subsequently influenced the outcome of various choice tests, the observed departures from random mating do not suggest strong or consistent sexual isolation among the strains. It is clear that a significant amount genetic differentiation in *D. mettleri* does not indicate that premating reproductive isolation occurs in allopatric populations. Perhaps some ecological factors such as host shifts, resource distribution, and/or abundance of competitor species play an undetected role in the courtship behavioral differences among *D. mettleri* populations.

In Chapter Three, we examined reproductive output and sexual behavior differences among allopatric populations of *D. mettleri.* We discovered that the number of offspring produced by a single mating in *D. mettleri* had a significant variability

depending upon the geographic strain. The strain from Catalina Island produced only about one-quarter of the offspring produced by mainland populations. The reproductive output for females could be a quantitatively inherited trait. On the other hand, gene(s) on the X chromosome clearly influenced the male productivity. In addition significant variation in copulation time among allopatric populations of *D. mettleri* was observed, although it was not positively associated with reproductive output. Copulations in the Catalina Island strain lasted about one minute more than the continental populations. The low reproductive output and increase of mating time are a function of both sexes from this *D. mettleri* strain and indicated that in both traits, male and female have coevolved. Also, we suggested that reproductive output and mating time probably are traits with different reaction to species ecology during the incipient speciation. Finally, we speculated that reproductive biology differences among *D. mettleri* populations were driven by ecological factors such as host shift. Decrease in reproductive output and increase in mating time in the *D. mettleri* Catalina Island population could be a response to the size reduction and chemical composition changes in the novel host *Opuntia*, which created a high interspecific competition for larval resources.

Finally, in Chapter Four we used molecular methods to determine the degree of genetic differentiation among populations of *D. mettleri*, and we checked if both techniques showed similar results. Additionally, we explored evidence of postzygotic isolation and its potential association with genetic differentiation among *D. mettleri* populations. *Drosophila mettleri* from Catalina Island is significantly differentiated genetically from all localities compared, both using alloenzyme electrophoresis and

*m*DNA analyses. In addition, a notable genetic differentiation was found within Baja populations. On the other hand, an important percentage of F_1 male sterility occurred mainly among population crosses involving individuals from Catalina Island. We considered this situation as an early form of postzygotic isolation.

Overall, while strain differences for many traits indicated the existence of genetic differences among *D. mettleri* populations, only male sterility exhibited some evidence of reproductive isolation. In fact, male sterility involved the Santa Catalina Island population, which also showed the greatest genetic differentiation as evidenced in both the allozyme and *m*DNA data. Therefore, in *D. mettleri*, at least, it appears that postmating isolation has developed before premating isolation.

In conclusion, it is clear that we need to know the genetic basis of reproductive isolation in order to recognize the initial steps in the speciation. With *D. mettleri* as a model of speciation, there are several unanswered subjects such as preference-performance, climatic limitations, interspecific competition and chemical adaptations among populations that may provide clues to understand how populations changes prior to the formation of reproductive barriers. Therefore, *D. mettleri* as speciation model may help to increase our knowledge, not only about the genetics, but also the ecological circumstances that facilitate an incipient speciation.

APPENDIX A: ILLUSTRATIONS

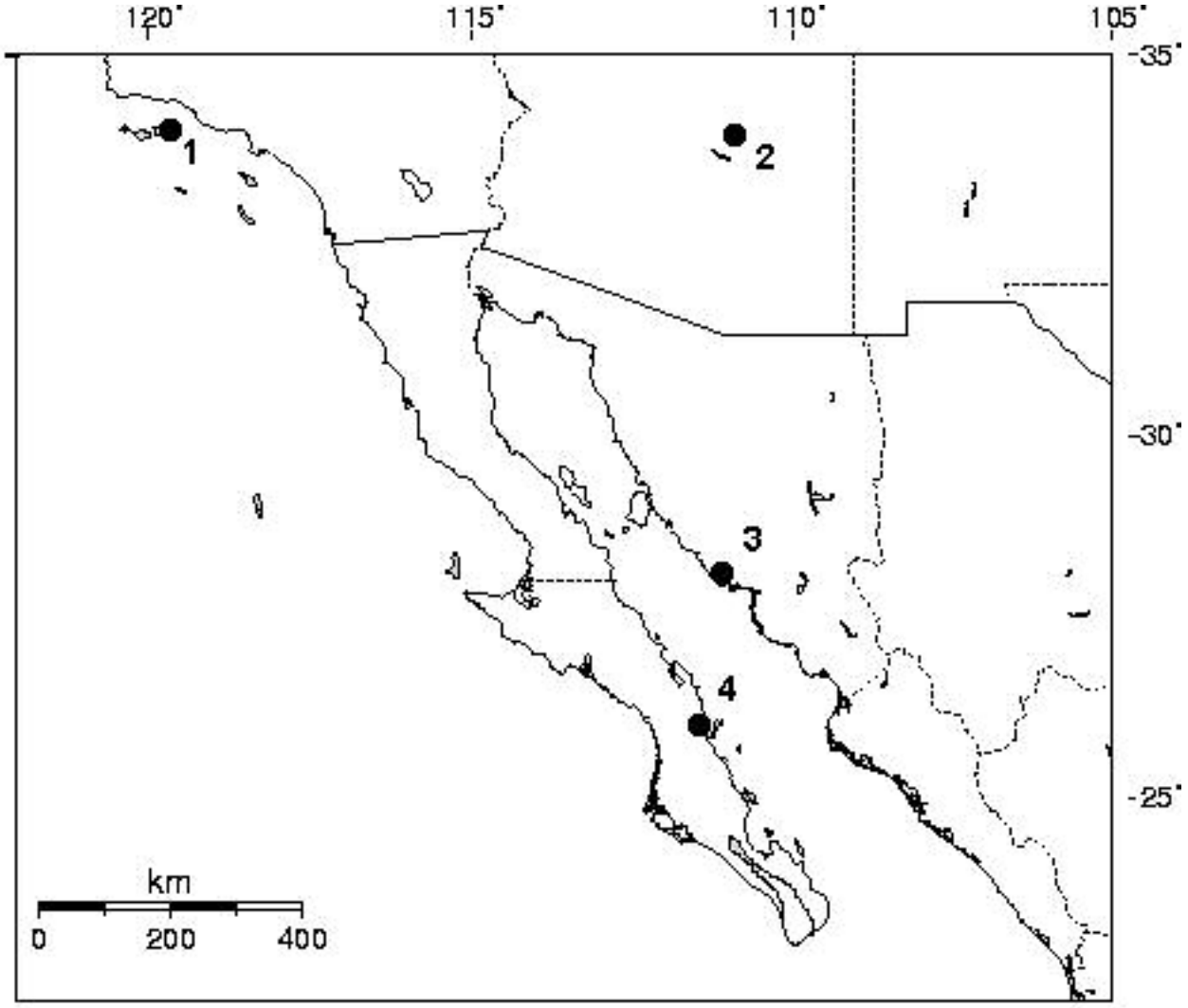

Figure 1. Key to the *Drosophila mettleri* populations used in chapter two and three: 1 = Santa Catalina island, CA (CAT); 2 =Superstition Mountains, AZ (SUP); 3 = San Jose de Guaymas, Guaymas, Sonora, Mexico (GYM); 4 = Bahía Concepción, Loreto, Baja California South, Mexico (LO).

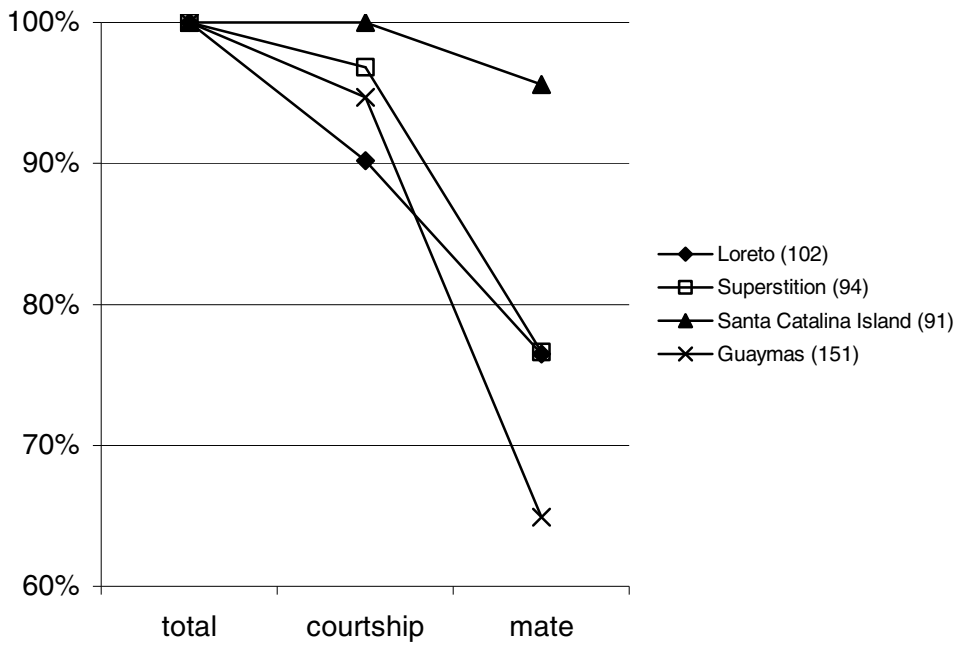

Figure 2. Courtship and mating frequencies for intrapopulation pairs in four strains of *Drosophila mettleri*.

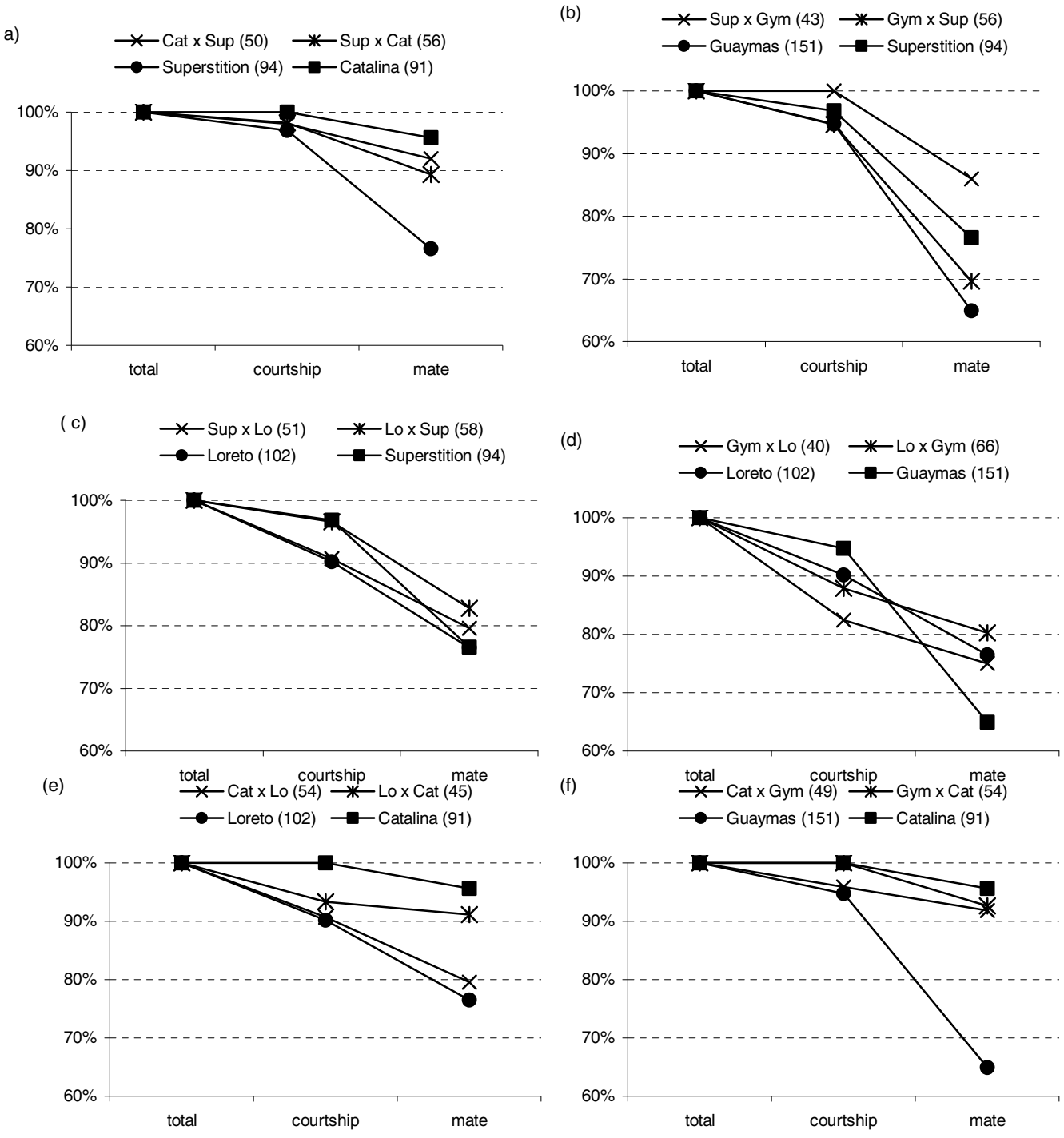

Figure 3. Courtship and mating frequencies for interpopulation pairs in four strains of *Drosophila mettleri*: (a) CAT & SUP, (b) GYM & SUP, (c) LO & SUP, (d) LO & GYM, (e) LO & CAT, (f) GYM & CAT. Crosses are listed as female X male.

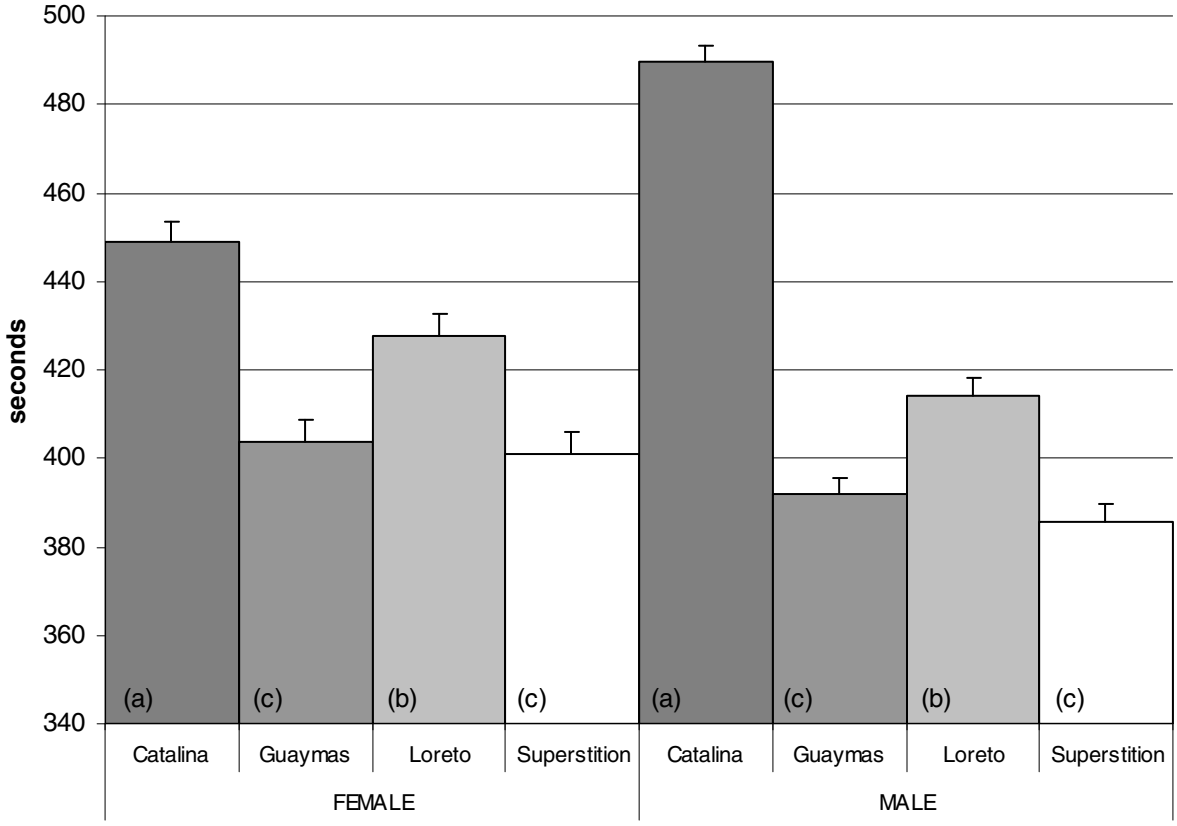

Figure 4. *Copulation duration.* Average mating duration in four populations of *Drosophila mettleri* by gender (seconds). Statistical significant groups marked at the base of each bar.

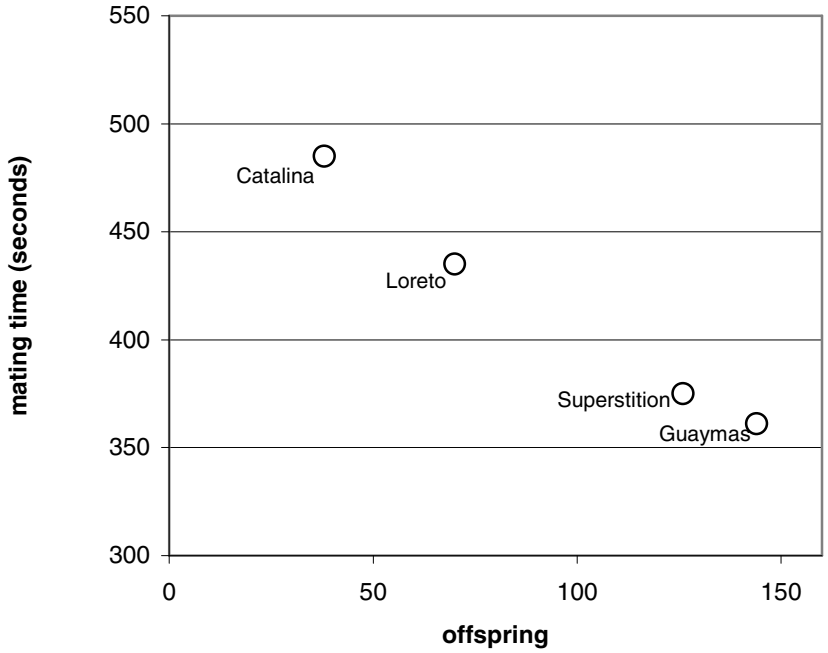

Figure 5. Average mating duration (Y-axis) and average reproductive output (X-axis) in four populations of *Drosophila mettleri*.

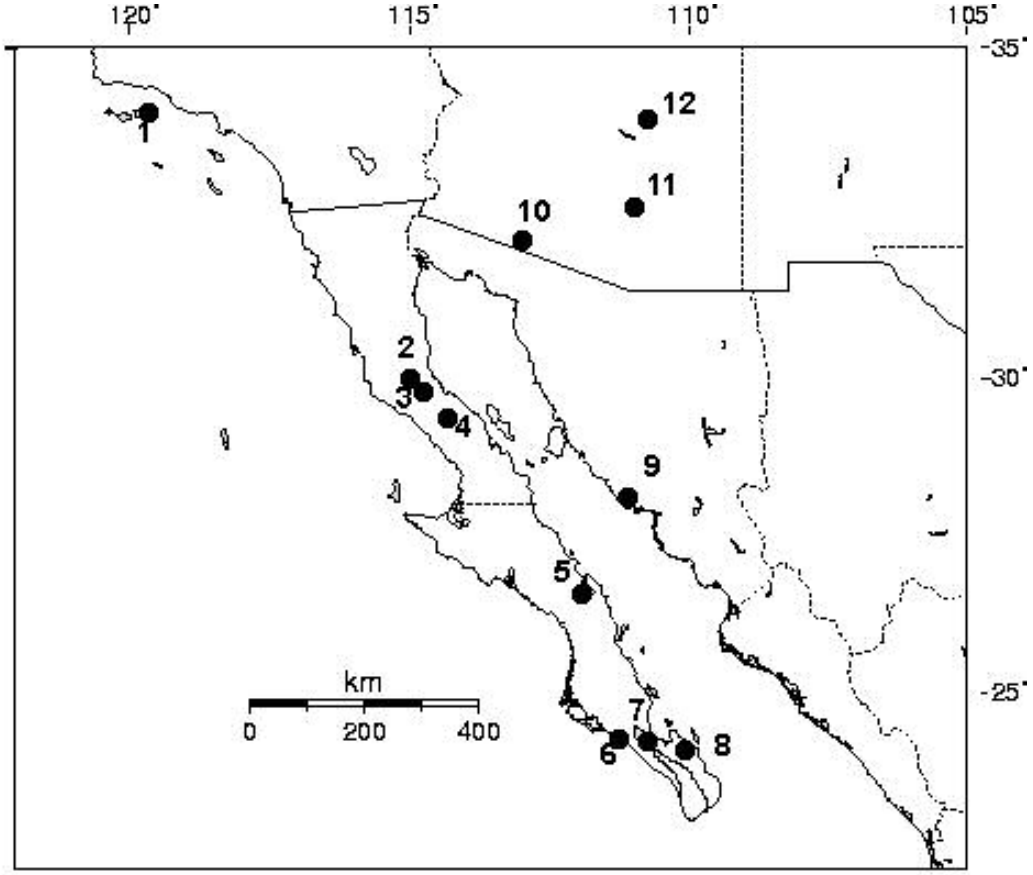

Fig. 6. Key to the populations of *Drosophila mettleri* sampled for chapter four: 1) Santa Catalina Island, CA; 2) Cataviña, BC; 3) Lago Chapala, BC; 4) Highway 1 249 km; BC, 5) Bahia Conception, BCS; 6) Punta Conejo, BCS; 7) El Cien, BCS; 8)Ensenada de los Muertos, BCS; 9) Guaymas, Sonora; 10) Organ Pipe National Monument, AZ; 11) Tucson, AZ; and 12) Sierra Ancha, AZ.

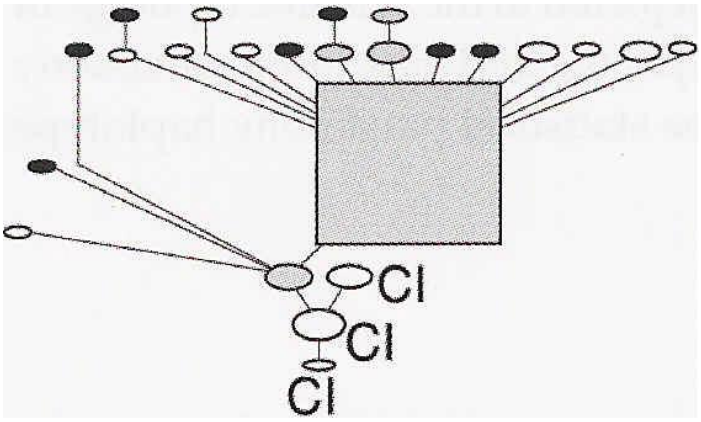

Figure 7. Statistical parsimony haplotypes network in eight populations *of Drosophila mettleri.* Ancestral haplotypes determined by TSC are represented in the square. Haplotypes observed only in peninsular individuals shown in white ovals. Black ovals show haplotypes unique to continental individuals. Grey ovals are haplotypes observed in peninsular and continental individuals. Exclusive haplotypes on insular individuals are marked with CI.

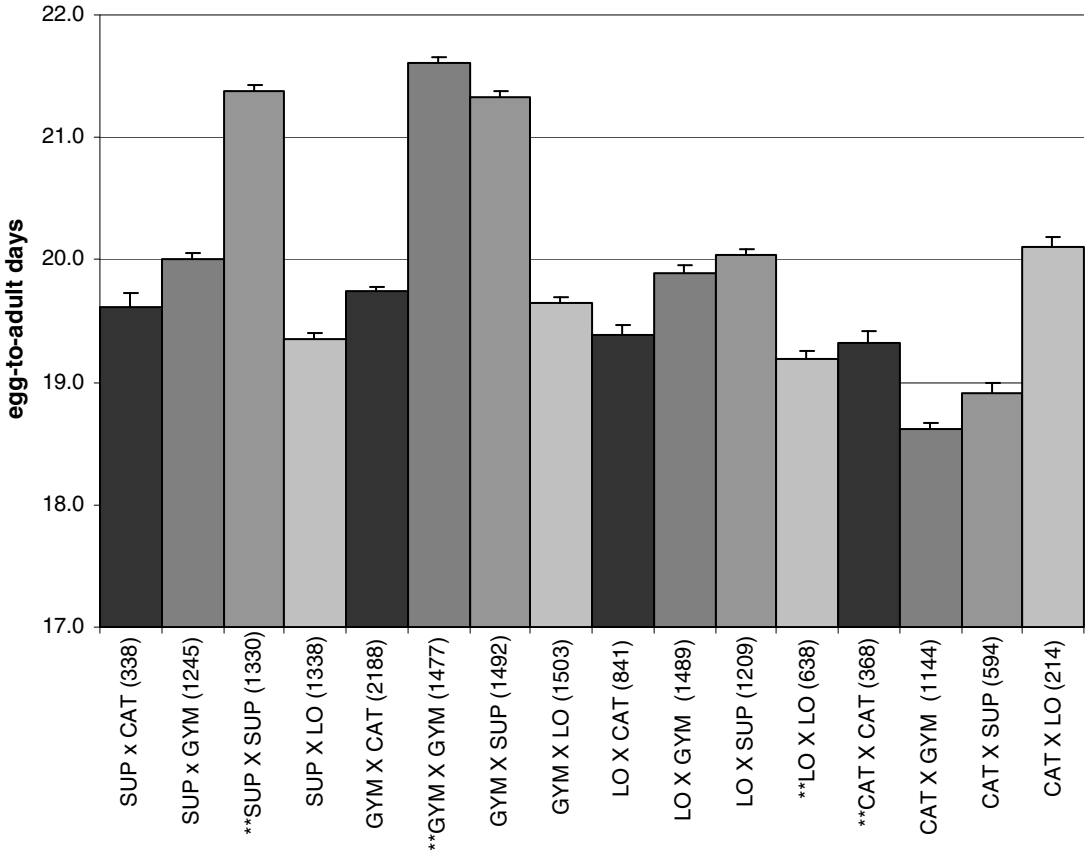

Figure 8. Postzygotic isolation in *Drosophila mettleri*: average development time in days. Populations are designed as follow: Santa Catalina Island, designated "CAT"; Superstition Mountains, designated "SUP"; Loreto, designated "LO"; and Guaymas designated "GYM". Numbers in parenthesis indicate (n) value of individuals used. Combinations marked "**" indicate control lines.

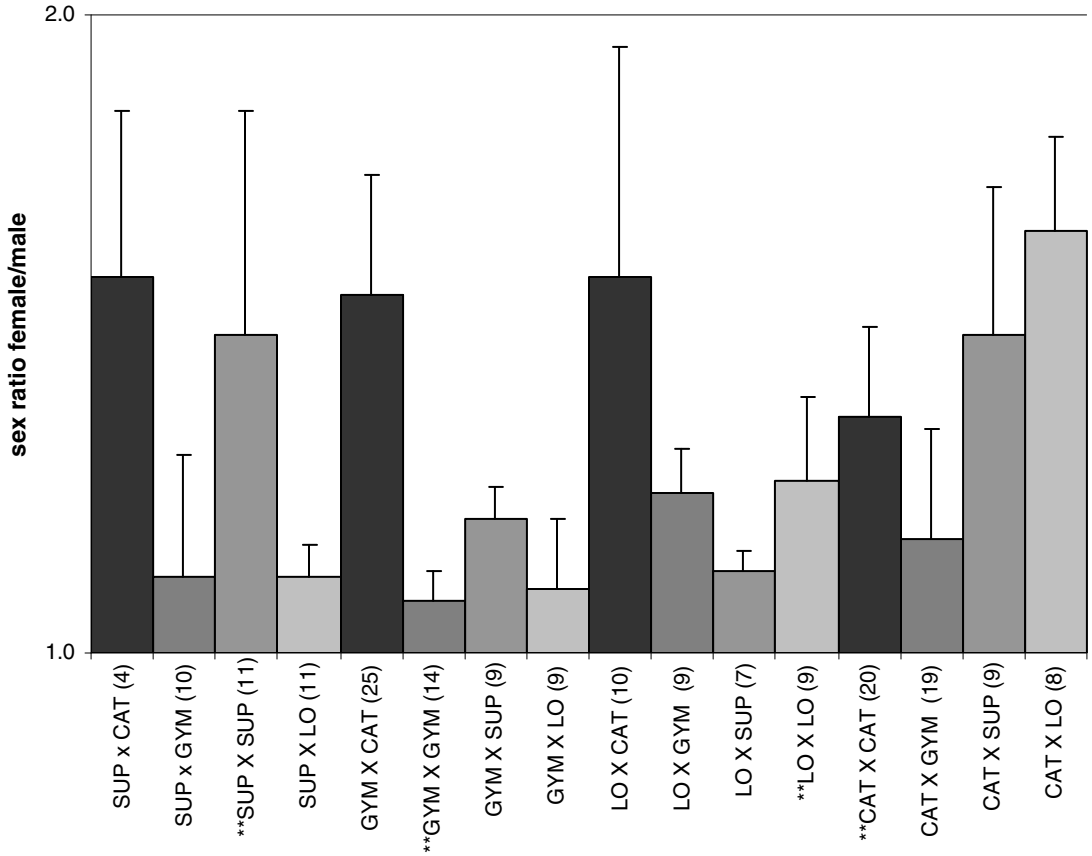

Figure 9. Postzygotic isolation in *Drosophila mettleri*: sex ratio (female/males). Populations are designed as follow: Santa Catalina Island, designated "CAT"; Superstition Mountains, designated "SUP"; Loreto, designated "LO"; and Guaymas designated "GYM". Numbers in parenthesis indicate (n) value of lines used. Combinations marked "**" indicate control lines.

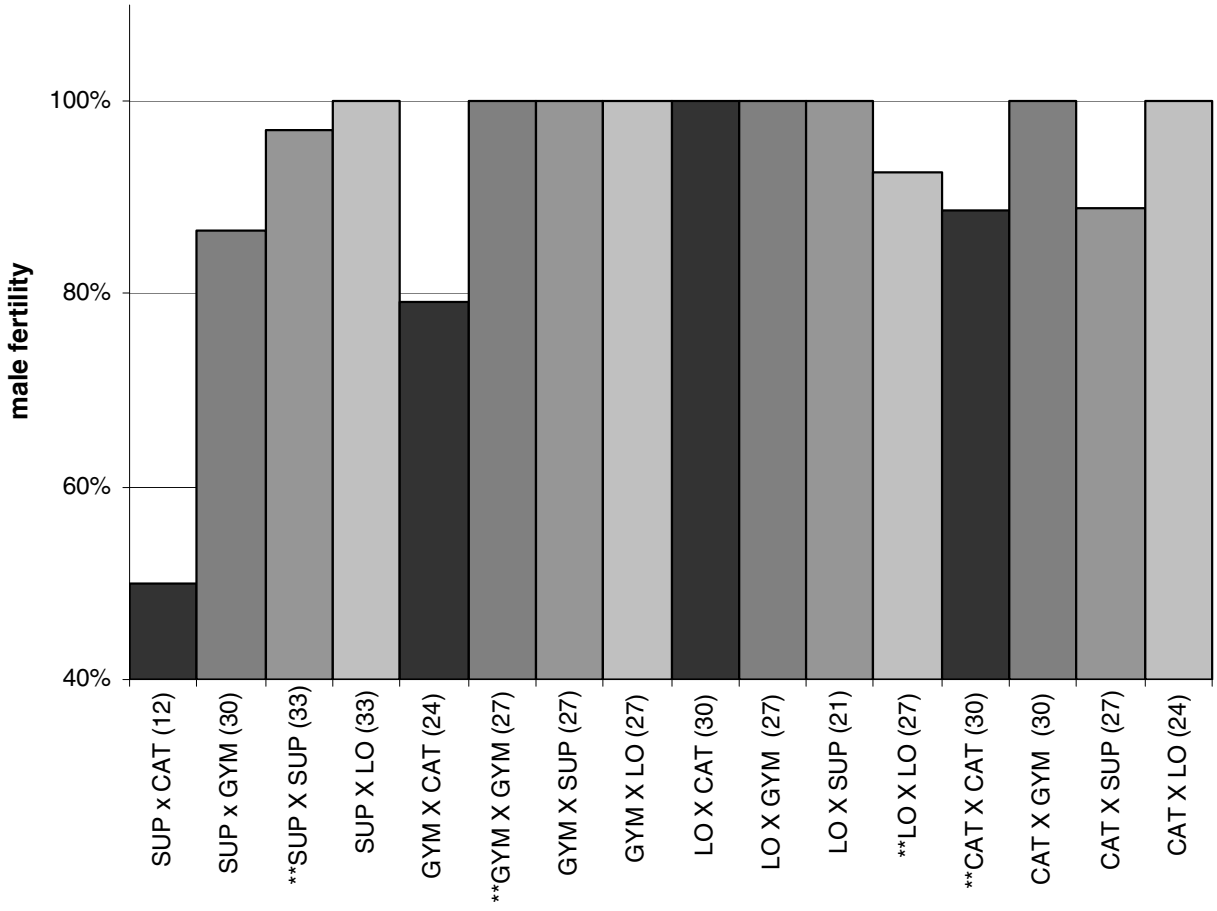

Figure 10.Postzygotic isolation in *Drosophila mettleri*: male fertility. Populations are designed as follow: Santa Catalina Island, designated "CAT"; Superstition Mountains, designated "SUP"; Loreto, designated "LO"; and Guaymas designated "GYM". Numbers in parenthesis indicate (n) value of males dissected. Combinations marked "**" indicate control lines.

APPENDIX B: TABLES

Table 1. *No choice test*. Male latency, female receptivity, and overall mating speed in four strains of *Drosophila mettleri* (in seconds). Results for intrapopulation and interpopulation combinations.

♀ x ♂	(n)	**Male latency X ± S.E. (n)**	**Female receptivity X ± S.E. (n)**	**Mating speed X ± S.E. (n)**
Intrapopulation pairs				
CAT x CAT	74	46.4 ± 5.1 [a,b,c]	35.0 ± 4.4 [a,b,c]	81.3 ± 6.1 [a,b,c]
SUP x SUP	61	42.1 ± 5.0 [a,b,c]	46.7 ± 6.4 [a,b,c]	88.9 ± 7.4 [a,b,c,d]
GYM x GYM	87	56.3 ± 4.1 [a,b,c]	56.1 ± 6.9 [b,c]	112.3 ± 7.1 [a,b,c,d]
LO x LO	61	55.2 ± 4.7 [a,b,c]	23.5 ± 4.3 [a,b]	78.8 ± 5.9 [a,b,c]
Interpopulation pair				
SUP x CAT	40	45.7 ± 5.0 [a,b,c]	44.4 ± 6.3 [a,b,c]	90.1 ± 7.2 [a,b,c,d]
GYM x CAT	45	52.4 ± 5.4 [a,b,c]	42.4 ± 5.7 [a,b,c]	94.8 ± 7.2 [a,b,c,d]
LO x CAT	32	37.8 ± 4.6 [a,b,c]	45.8 ± 8.0 [a,b,c]	83.5 ± 8.6 [a,b,c,d]
CAT x SUP	35	38.2 ± 5.2 [a,b,c]	34.8 ± 5.1 [a,b,c]	73.0 ± 6.4 [a,b,c]
GYM x SUP	33	56.0 ± 7.3 [a,b,c]	62.9 ±13.9 [b,c]	118.9 ± 15.7 [a,b,c,d,e]
LO x SUP	39	77.6 ± 10.3 [b,c,d]	30.3 ± 5.6 [a,b,c]	107. 9 ±10.4 [a,b,c,d]
CAT x GYM	40	43.0 ± 7.0 [a,b,c]	39.9 ± 6.6 [a,b,c]	82.9 ± 8.4 [a,b,c,d]
SUP x GYM	29	31.3 ± 3.0 [a,b]	63.7 ± 13.8 [b,c]	95.0 ± 14.5 [a,b,c,d]
LO x GYM	41	56.8 ± 7.4 [a,b,c]	32.1 ± 4.5 [a,b,c]	88.9 ± 8.1 [a,b,c,d]
CAT x LO	30	113.8 ± 20.6 [c,d,e]	19.7 ± 3.0 [a,b]	133.5 ± 20.6 [b,c,d,e]
SUP x LO	31	129.8 ± 23.9 [d,e]	43.8 ± 10.2 [a,b,c]	173.5 ± 23.0 [c,d,e]
GYM x LO	26	106.9 ± 15.6 [c,d,e]	19.1 ± 4.4 [a,b]	126.0 ±15.6 [a,b,c,d,e]

Table 2. Two factor analysis of variance and effects tests in NO CHOICE TESTS for a) male latency, b) female receptivity, and c) overall mating speed in four populations of *Drosophila mettleri.* Analyses between and within populations.

a)

Analysis of variance

Source	Df	Sum of squares	Mean Square	F ratio	P value
Model	15	420688.6	28045.9	9.5020	P<0.0001
Error	688	2030687.9	2951.6		
C. total	703	2451376.5			

Effect tests

Source	Df	Sum of squares	F ratio	P value
Female	3	10185.4	1.1503	0.3280
Male	3	300114.6	33.8931	P<0.0001
Female*male interaction	9	187629.1	7.0632	P<0.0001

b)

Analysis of variance

Source	Df	Sum of squares	Mean Square	F ratio	P value
Model	15	110451.7	7363.5	3.3211	P<0.0001
Error	688	1525410.6	2217.2		
C. total	703	1635862.4			

Effect tests

Source	Df	Sum of squares	F ratio	P value
Female	3	35420.2	5.3252	P<0.01
Male	3	37111.8	5.5795	P<0.001
Female*male interaction	9	28531.8	1.4298	0.1712

c)

Analysis of variance

Source	Df	Sum of squares	Mean Square	F ratio	P value
Model	15	363259.3	24217.3	5.4753	P<0.0001
Error	688	3043022.5	4423.0		
C. total	703	3406281			

Effect tests

Source	Df	Sum of squares	F ratio	P value
Female	3	137155.7	10.3366	P<0.01
Male	3	71525.6	5.3904	P<0.0001
Female*male interaction	9	187676.3	4.7147	P<0.0001

Table 3 Male choice test in four strains of *Drosophila mettleri*: (a) First female-courted and (b) female choose. Chi-square and isolation index are shown for each type of male combination.

(a)	Male	(n)	Homotypic	(n)	Heterotypic	(n)	χ^2
	CAT	41	CAT	15	SUP	26	3.0
	CAT	55	CAT	25	GYM	30	0.5
	CAT	45	CAT	20	LO	25	0.6
	SUP	41	SUP	18	CAT	23	0.6
	SUP	42	SUP	26	GYM	16	2.4
	SUP	43	SUP	24	LO	19	0.6
	GYM	46	GYM	17	CAT	29	3.1
	GYM	42	GYM	15	SUP	27	3.4
	GYM	52	GYM	22	LO	30	1.2
	LO	43	LO	24	CAT	19	0.6
	LO	43	LO	24	SUP	19	0.6
	LO	40	LO	15	GYM	25	2.5

(b)	Male	(n)		(n)		(n)	χ^2	I ± S.E.
	CAT	41	CAT	21	SUP	20	0.0	0.02 ± 0.15
	CAT	55	CAT	36	GYM	19	5.3*	0.31 ± 0.13*
	CAT	45	CAT	23	LO	22	0.0	0.02 ± 0.15
	SUP	41	SUP	19	CAT	22	0.2	-0.07 ± 0.16
	SUP	42	SUP	28	GYM	14	4.7*	0.33 ± 0.15*
	SUP	43	SUP	23	LO	20	0.2	0.07 ± 0.15
	GYM	46	GYM	21	CAT	25	0.4	-0.09 ± 0.14
	GYM	42	GYM	13	SUP	29	6.1*	-0.38 ± 0.14*
	GYM	52	GYM	22	LO	30	1.2	-0.15 ± 0.14
	LO	43	LO	17	CAT	26	1.9	0.21 ± 0.15
	LO	43	LO	22	SUP	21	0.0	0.02 ± 0.15
	LO	40	LO	15	GYM	25	2.5	-0.25 ± 0.15

Table 4. Female choice test in four strains of *Drosophila mettleri*: (a) First male to court and (b) male accepted to mate. Chi-square and isolation index are shown for each type of male combination.

(a)

Female	(n)	intrapopulation male	(n)	Interpopulation male	(n)	χ^2
CAT	41	CAT	28	SUP	15	3.0
CAT	42	CAT	22	GYM	20	0.1
CAT	40	CAT	27	LO	13	4.9*
SUP	42	SUP	15	CAT	27	3.4
SUP	40	SUP	16	GYM	24	1.6
SUP	40	SUP	31	LO	9	12.1*
GYM	42	GYM	23	CAT	19	0.4
GYM	43	GYM	26	SUP	17	3.6
GYM	42	GYM	38	LO	4	27.5*
LO	40	LO	17	CAT	23	0.9
LO	45	LO	12	SUP	33	9.8*
LO	41	LO	5	GYM	36	23.4*

(b)

Female	(n)	intrapopulation male	(n)	Interpopulation male	(n)	χ^2	I ± S.E.
CAT	41	CAT	25	SUP	16	1.9	0.22 ± 0.15
CAT	42	CAT	20	GYM	22	0.1	-0.05 ± 0.15
CAT	40	CAT	27	LO	13	4.9*	0.35 ± 0.15*
SUP	42	SUP	20	CAT	22	0.1	-0.05 ± 0.15
SUP	40	SUP	14	GYM	26	3.6*	-0.30 ± 0.15*
SUP	40	SUP	31	LO	9	12.1*	0.55 ± 0.13*
GYM	42	GYM	23	CAT	19	0.4	0.10 ± 0.15
GYM	43	GYM	25	SUP	15	2.5	0.23 ± 0.15
GYM	42	GYM	38	LO	4	27.6*	0.81 ± 0.09*
LO	40	LO	23	CAT	17	0.9	0.15 ± 0.16
LO	45	LO	14	SUP	31	6.4*	-0.38 ± 0.14*
LO	41	LO	5	GYM	36	23.4*	-0.76 ± 0.10

Table 5. Multiple choice tests in four strains of *Drosophila mettleri.* Chi-square and isolation index are shown for each type combination.

Population A	x	Population B	Matings Observed(n)	Ax A	A x B	A x B	B x B	χ^2	I ± S.E.
SUP	x	LO	77% 185/240	50	35	56	44	5.21	0.02 ± 0.07
SUP	x	GYM	86% 206/240	47	62	50	47	2.97	-0.09 ± 0.07
SUP	x	CAT	77% 184/240	31	53	44	56	8.22*	-0.05 ± 0.07
LO	x	CAT	85% 203/240	31	64	40	68	19.29**	-0.02 ± 0.07
CAT	x	GYM	81% 195/240	61	41	52	51	5.76	0.05 ± 0.07
LO	x	GYM	79% 190/240	46	43	46	55	1.71	0.06 ± 0.07

* $p<0.05$
** $p<0.01$

Table 6. Reproductive output matings from four geographic strains of *D. mettleri.* a) Intrapopulation crosses, b) interpopulation crosses, c) summary of female output across male strains and, d) summary of male output across female strains. Statistical groups are shown in the average offspring column.

females	males	Productive mating (%) (n/N)	Total offspring	Average Offspring / Female
a) Intrapopulation crosses				
Superstition	Superstition	91.7 (11/12)	1330	126.5 ± 21.3^{abc}
Guaymas	Guaymas	56.0 (14/25)	2017	144.1 ± 27.4^{ab}
Loreto	Loreto	75.0 (9/12)	635	70.6 ± 21.2^{abc}
Santa Catalina Island	Santa Catalina Island	80.0 (20/25)	752	37.6 ± 6.6^{c}
b) Interpopulation crosses				
Superstition	Loreto	91.7 (11/12)	1338	121.6 ± 17.3^{abc}
Superstition	Santa Catalina Island	33.3 (4/12)	338	84.5 ± 37.7^{abc}
Superstition	Guaymas	83.3 (10/12)	1425	142.5 ± 21.4^{ab}
Guaymas	Superstition	75.0 (9/12)	1492	165.8 ± 21.0^{ab}
Guaymas	Loreto	75.0 (9/12)	1503	167.0 ± 36.4^{ab}
Guaymas	Santa Catalina Island	63.0 (17/27)	1831	107.7 ± 19.6^{bc}
Loreto	Santa Catalina Island	83.3 (10/12)	841	84.1 ± 23.3^{abc}
Loreto	Superstition	58.3(7/12)	1219	172.7 ± 15.7^{ab}
Loreto	Guaymas	75.0 (9/12)	1489	165.4 ± 31.6^{ab}
Santa Catalina Island	Loreto	66.7 (8/12)	514	64.3 ± 19.6^{abc}
Santa Catalina Island	Guaymas	76.0 (19/24)	1144	60.2 ± 10.0^{abc}
Santa Catalina Island	Superstition	75.0 (9/12)	594	66.0 ± 19.0^{abc}
c) Female crosses				
Santa Catalina Island	-	76.7 (56/73)	3004	52.7 ± 10.7
Guaymas	-	64.5 (49/76)	6843	134.3 ± 11.1
Loreto	-	72.9 (35/48)	4184	119.3 ± 11.8
Superstition	-	75.0 (36/48)	4431	124.8 ± 12.6
d) Male crosses				
-	Santa Catalina Island	67.1 (51/76)	3762	54.4 ± 12.5
-	Guaymas	71.2 (52/73)	6075	128.8 ± 10.7
-	Loreto	77.1 (37/48)	3990	127.8 ± 11.4
-	Superstition	75.0 (36/48)	4635	130.4 ± 11.6

Table 7. *Reproductive output.* Two factor analysis of variance and effects tests for *Drosophila mettleri:* Analysis between and within populations.

Analysis of variance

Source	Df	Sum of squares	Mean Square	F ratio	P value
Model	15	359054.8	23937.0	5.0388	P<0.001
Error	142	674576.6	4750.5		
C. total	157	1033631.4			

Effect tests

Source	Df	Sum of squares	F ratio	P value
Female	3	135591.2	9.5141	P<0.001
Male	3	101250.0	7.1045	P<0.01
Female*male interaction	9	79858.8	1.8678	0.0614

Table 8. The influence of the X chromosome on male reproductive output between the Santa Catalina Island and Guaymas strains of *D. mettleri.*

	females	males	Average Offspring/Female (N mating)
a) Intrapopulation			
	Guaymas	Guaymas	144.1 ± 27.4(14)
	Santa Catalina Island	Santa Catalina Island	37.6 ± 6.6 (20)
b) Interpopulation			
	Guaymas	Santa Catalina Island	107.7 ± 19.6 (17)
	Santa Catalina Island	Guaymas	60.2 ± 10.0 (19)
c) Backcrosses			
	Santa Catalina Island	Santa Catalina Island x Guaymas	59.3 ± 7.0 (29)
	Santa Catalina Island	Guaymas x Santa Catalina Island	65.6 ± 14.5 (14)
	Guaymas	Santa Catalina Island x Guaymas	77.7 ± 7.9 (26)
	Guaymas	Guaymas x Santa Catalina Island	153.2 ± 26.5 (14)

Table 9. *Copulation duration* in four strains of *Drosophila mettleri* (data in seconds): a) Intrapopulation crosses and b) interpopulation crosses. Statistical groups are shown in the mating duration column.

Female	Male	(n)	Mating duration X ± S.E. (n)
a) Intrapopulation pairs			
Superstition	Superstition	62	361.9 ± 5.1^{efg}
Guaymas	Guaymas	76	375.0 ± 5.1^{defg}
Loreto	Loreto	64	434.5 ± 4.7^{bc}
Santa Catalina Island	Santa Catalina Island	75	484.5 ± 7.2^{a}
b) Interpopulation pairs			
Superstition	Loreto	29	355.1 ± 6.2^{efg}
Superstition	Santa Catalina Island	40	487.6 ± 7.2^{a}
Superstition	Guaymas	26	408.8 ± 7.4^{bcdef}
Guaymas	Superstition	33	373.7 ± 8.3^{defg}
Guaymas	Loreto	24	395.4 ± 6.1^{bcdefg}
Guaymas	Santa Catalina Island	38	492.7 ± 11.2^{a}
Loreto	Santa Catalina Island	31	500.7 ± 7.47^{a}
Loreto	Superstition	38	405.3 ± 8.1^{bcdef}
Loreto	Guaymas	44	386.1 ± 5.1^{defg}
Santa Catalina Island	Loreto	40	435.1 ± 7.8^{bcd}
Santa Catalina Island	Guaymas	35	424.5 ± 7.7^{bcd}
Santa Catalina Island	Superstition	38	415.3 ± 5.2^{bcde}

Table 10. Two factor analysis of variance and effects tests for mating duration in four populations of *Drosophila mettleri.*

Analysis of variance

Source	Df	Sum of squares	Mean Square	F ratio	P value
Model	15	1557985.8	103866	48.9191	P<0.0001
Error	678	1439539.2	2123		
C. total	693	2997525.0			

Effect tests

Source	Df	Sum of squares	F ratio	P value
Female	3	142483.8	22.3692	P<0.0001
Male	3	1100237.2	172.7314	P<0.0001
Female*male interaction	9	170995.5	8.9485	P<0.0001

Table 11. Summary of genetic variability at eight enzyme loci in populations of *Drosophila mettleri.*

Locality	H_o[2]	H_e[3]	Alleles per locus (mean ± SE)	P (95%)[4]
Guaymas[1]	0.125 (±0.069)	0.112 (±0.058)	2.63 (±0.63)	50.0
Organ Pipe Natl. Monument[1]	0.094 (±0.057)	0.086 (±0.050)	1.88 (±0.35)	12.5
Tucson[1]	0.104 (±0.050)	0.111 (±0.056)	2.63 (±0.46)	37.5
Santa Catalina Island	0.119 (±0.066)	0.101 (±0.055)	1.63 (±0.26)	25.0
Catavina	0078 (±0.050)	0.083 (±0.055)	1.63 (±0.32)	25.0
Highway1, km249	0.086 (±0.086)	0.062 (±0.062)	1.13 (±0.13)	12.5
Bahia Concepcion	0.113 (±0.061)	0.126 (±0.060)	1.63 (±0.18)	37.5
Ensenada de los Muertos	0.082 (±0.074)	0.078 (±0.063)	1.25 (±0.16)	25.0

[1] Data from Pfeiler and Markow (2001).

[2] Observed heterozygosity (direct count)

[3] Hardy Weinberg expected heterozygosity, unbiased estimate (Nei 1978).

[4] Percent polymorphic loci (95% criterionO).

Table 12. Summary of Wright's (1978) *F*-statistics[1] in polymorphic loci in eight populations[2] of *Drosophila mettleri*.

Locus	F_{IS}	F_{IT}	F_{ST}
Pgm	-0.137	0.520	0.578
Adh	-0.123	-0.009	0.102
Mdh-1	-0.011	-0.001	0.010
Gpdh	0.415	0.443	0.048
Pep-A	-0.085	-0.023	0.058
Pep-B	0.188	0.206	0.021
Est-2	-0.044	-0.022	0.021
Mean	-0.073	0.136	0.194

[1] Data from Pfeiler and Markow (2001).

[2] Eight populations are the same as shown in Table 1.

Table 13. Pairwise comparisons of F_{ST} in populations of *Drosophila mettleri*: a) electrophoresis alloenzyme, and b)*mtDNA* Asterisks indicate statistically significant differences at the 5% level.

a)

	Guaymas 1	Organ Pipe Natl. Monument 2	Tucson 3	Santa Catalina Island 4	Catavina 5	Highway 1, km249 6	Bahia Concepcion 7	Ensenada de los Muertos 8
1	-							
2	0.01	-						
3	-0.00	0.01	-					
4	0.42*	0.53*	0.38*	-				
5	0.08*	0.17*	0.11*	0.45*	-			
6	-0.03	-0.01	-0.02	0.54*	0.06*	-		
7	0.01	0.05*	-0.01	0.41*	0.04*	-0.05	-	
8	0.03*	0.09*	0.06*	0.48*	0.02	0.00	0.02	-

b)

	Guaymas 1	Sierra Ancha 2	Tucson 3	Santa Catalina Island 4	Lago Chapala 5	Punta Conejo 6	Bahia Concepcion 7	El Cien 8
1	-							
2	0.04	-						
3	0.02	0.00	-					
4	0.67*	0.76*	0.71*	-				
5	0.01	0.00	0.00	0.69*	-			
6	0.00	0.15*	0.13*	0.63*	0.11*	-		
7	0.10*	0.06*	0.05*	0.74*	0.03	0.23*	-	
8	0.05	0.07	0.00	0.81*	0.00	0.29*	0.05	-

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