Abstract

Title of Dissertation: POST-COPULATORY SEXUAL SELECTION AND GAMETIC ISOLATION IN STALK-EYED FLIES

Emily G. Rose, Doctor of Philosophy, 2011

Directed by: Dr. Gerald S. Wilkinson, Professor
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Understanding the forces that drive lineage splitting, i.e. speciation, has been a goal of evolutionary research since Darwin but remains poorly understood. Sexual selection is frequently invoked as a possible explanation, but focus is typically placed on precopulatory activities where males compete for access to females or females choose among males. The possibility that postcopulatory sexual selection, a powerful evolutionary force which involves interactions between sperm and the female reproductive tract, may contribute to reproductive isolation has only recently been considered. Using diopsid stalk-eyed flies as a model system, I examine divergence in fertilization systems among closely related populations of a single species (*Teleopsis dalmanni*), in order to assess whether gametic isolation has the potential to contribute to speciation.

In chapter 2, I measure a suite of reproductive and non-reproductive morphological traits in eight closely related populations to determine their relative rates of evolution. I find that reproductive traits have diverged more rapidly than non-reproductive traits and that male and female postcopulatory traits, i.e. sperm length and sperm storage organ dimensions, have coevolved.

Chapters 3 and 4 describe experiments aimed at elucidating the importance of gametic isolation among these populations. Chapter 3 is an examination of non-competitive gametic isolating barriers. I performed 275 crosses between four populations and measured mechanisms of non-competitive gametic isolation including sperm transfer, sperm survival, sperm motility and ability of sperm to reach the site of fertilization. I conclude that non-competitive gametic isolation exists among these population pairs and specifically identify the inability of sperm to reach the site of fertilization in between-population crosses as a mechanism of reproductive isolation.

Chapter 4 is an investigation of competitive gametic isolation which occurs when sperm of males from different populations compete for fertilization. Using two pairs of populations, I carry out every possible combination of crosses and genotype over 1200 offspring to determine paternity. The results demonstrate that sperm competition further inhibits successful hybridization among these closely related populations.

I conclude that postcopulatory sexual selection and gametic isolation have the potential to play an important role in the formation of new species in this system.

POSTCOPULATORY SEXUAL SELECTION AND GAMETIC ISOLATION IN STALK-EYED FLIES

By

Emily G. Rose

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Advisory Committee
Dr. Gerald S. Wilkinson, Chair
Dr. Gerald Borgia
Dr. Eric Haag
Dr. Charles Mitter
Dr. Karen Carleton

Dedication

For my family and in loving memory of my father Alan

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Chapter 1: Introduction to dissertation

Theoretical Background

Over the past several decades, research and theory on sexual selection has been expanded to include events that occur after copulation but before fertilization and involve interactions between sperm or seminal products produced by males and female reproductive tracts (Parker 1970). More recently, interest in the power of these postcopulatory interactions to create barriers to gene flow among populations has arisen (Eady 2001; Howard 1999). Such barriers have traditionally been neglected by evolutionary biologists, as demonstrated by the conventional division of reproductive isolating barriers into premating and postzygotic. My dissertation research examines whether postcopulatory traits have diverged among closely related allopatric populations of stalk-eyed flies (chapter 2) and whether such changes have driven the evolution of reproductive isolation in the form of non-competitive (chapter 3) and competitive (chapter 4) gametic isolation.

Sexual selection occurs when reproduction of individuals is influenced by differential access to mates or gametes. The two processes which cause sexual selection are intrasexual competition for access to members of the opposite sex, and intersexual discrimination or choice among members of the opposite sex (Darwin 1871). Generally, these processes take the form of male-male competition and female choice. Females of many species mate with multiple males and have the capacity to store sperm, thus interactions between the sexes often continue beyond the acts of mate acquisition and copulation (Parker 1970). In this postcopulatory context, female

behavior, reproductive tract morphology, and physiology potentially allow the female to cryptically "choose" among the sperm of multiple males while sperm compete within the female reproductive tract for access to eggs.

Postcopulatory sexual selection can be a powerful evolutionary force, capable of driving rapid evolution of reproductive characters and producing an astounding variety of morphological, physiological, and behavioral adaptations (Birkhead and Moller 1998; Eberhard 1996; Eberhard and Cordero 1995; Meiklejohn et al. 2003; Pitnick et al. 1999; Simmons 2001). Traits ranging from behavioral rejection of copulations to reproductive tract morphology and biochemical gametic interactions are known to play a role in sperm competition and cryptic female choice (Eberhard 1996; Simmons 2001).

In many systems, male and female morphological characters involved in postcopulatory sexual selection coevolve (reviewed in Eberhard 1996). For example, comparative studies in beetles (Dybas and Dybas 1981), stalk-eyed flies (Presgraves et al. 1999), moths (Morrow and Gage 2000), dung flies (Minder et al. 2005), and passerine birds (Briskie et al. 1997) have all found a significant, positive relationship between the size of the sperm storage organ and sperm traits across species. Additionally, male genitalic structures show rapid and divergent evolution and coevolve with female reproductive tract morphologies in a variety of taxa (Arnqvist 1998). Recent studies show a more rapid accumulation of morphological divergence, levels of polymorphism, and sequence divergence in postcopulatory characters than other types of characters (Alipaz et al. 2001; Civetta and Singh 1998; Ramm et al. 2009). Proteins involved in postcopulatory interactions also show a signature of

positive selection at the DNA level in a wide array of taxa ranging from marine invertebrates to primates (Aagaard et al. 2010; Swanson et al. 2001; Vacquier 1998; Wyckoff et al. 2000; Zhang et al. 2007). Several studies suggest that this rapid, correlated evolution of male and female reproductive traits plays a causal role in reproductive isolation (Gavrilets 2000; Parker and Partridge 1998; Pitnick et al. 2003b; Rice 1998).

While the role of pre-copulatory male-male competition and female choice in creating exaggerated traits and potentially resulting in speciation has been explored extensively (Andersson 1994; Coyne and Orr 2004), the degree to which coevolution of postcopulatory characters plays a role in fueling speciation has been a subject of debate (Arnqvist 1998; Eady 2001; Gavrilets 2000; Panhuis et al. 2001; Pitnick et al. 2003b; Rice 1998). Dobzhansky (1937) first recognized that incompatibility at the gametic level could contribute to reproductive isolation. However this idea was largely overlooked until the 1990's (Coyne and Orr 2004).

Gametic isolation can occur in two forms: non-competitive and competitive. Non-competitive gametic isolation occurs when heterospecific or heteropopulation sperm are unable to achieve fertilization in the absence of sperm competition. This process has the potential to occur at any stage between copulation and fertilization. For example, poor transfer and storage of sperm, inviability or decreased motility of sperm in the female reproductive tract, inability of sperm and egg to fuse, or failure to stimulate oviposition are all known mechanisms of non-competitive gametic isolation (Coyne and Orr 2004). The most compelling evidence of non-competitive gametic isolation comes from externally spawning organisms, in which divergence in the

sperm-egg fusion reaction has caused reproductive incompatibilities. Studies of the evolution of sperm and egg recognition molecules in abalones have found that these molecules are highly species specific and evolve rapidly by positive selection (Aagaard et al. 2010; Kresge et al. 2001). Broadcast spawning is a simplified fertilization system; in internal fertilizers males and their gametes face the added challenge of successful sperm transfer, navigating the often convoluted ducts of the female reproductive tract (Eberhard 1996), and stimulating oviposition.

Competitive gametic isolation occurs when conspecific and heterospecific sperm compete for fertilization. In this scenario, sperm from each male type is physiologically capable of fertilizing the ova but sperm competition results in a fertilization advantage for one male type, most commonly the conspecific male (Gregory and Howard 1994). This pattern, referred to as conspecific sperm precedence, has been well documented (Chang 2004; Dixon et al. 2003; Geyer and Palumbi 2005; Gregory and Howard 1994; Howard 1999; Martin-Coello et al. 2009; Price 1997; Rieseberg et al. 1995; Wade et al. 1994). There are several evolutionary explanations that can be used to predict the outcome of such a cross. Cryptic sexual selection predicts that the conspecific male is best adapted to his mate and thus will sire the majority of her offspring. This outcome may be due to sperm competitive advantages of the conspecific male, cryptic female preference for the conpopulation male, or a combination of the two (Howard 1999). In contrast, sexual conflict theory predicts that males from closely related but different populations or species will have an advantage in sperm competition because females will not have evolved to resist their manipulations (Andres and Arnqvist 2001; Hosken et al. 2002; Rice 1998). This

advantage will come at some fitness cost to females. Only conspecific or conpopulation sperm precedence leads to clear reproductive barriers that can account for splitting lineages. If heterospecific sperm precedence occurs, then sperm competition would not create a barrier to hybridization among populations, and would enhance gene flow upon secondary contact resulting in genomic homogenization. Empirical findings more commonly support conspecific sperm precedence over heterospecific sperm precedence. However, some examples of heterospecific sperm precedence have been found (Hosken et al. 2002; Peterson et al. 2011; Tregenza and Wedell 2002).

Non-competitive and competitive gametic isolation are by no means mutually exclusive, and have been found to occur simultaneously in several systems (Brown and Eady 2001; Price 1997; Price et al. 2001).

Study System

Stalk-eyed flies (Diptera: Diopsidae) in the genus *Teleopsis* provide an ideal system for studying postcopulatory sexual selection and its potential importance for reproductive isolation because both males and females remate frequently in nature and in the lab, with females showing no reduction in receptivity after mating (Grant et al. 2002). Females store sperm from a single mating for up to 30 days, and when multiple males mate with a female within a population, paternity is equally shared among them, on average (Lorch et al. 1993). Additionally, seminal fluid can affect the outcome of sperm competition (Fry and Wilkinson 2004). These conditions indicate a

high probability of sperm mixing and the potential for sperm competition, cryptic female choice, and/or sexual conflict (Simmons 2001).

Female stalk-eyed flies have two primary sperm storage organs: the ventral receptacle (VR) and the spermathecae. In *Teleopsis* there are three sclerotized mushroom-shaped spermathecae which function in long-term sperm storage. The VR functions in short-term sperm storage, and is located nearer to the point of spermatophore deposit and the base of the oviduct, which is the site of fertilization (Kotrba 1993; Kotrba 1996). Sperm are transferred in a spermatophore (sperm packet), which is deposited by the male into the bursa copulatrix (Kotrba 1996). The contents of the spermatophore then migrate up the spermathecal ducts and into the spermathecae for long-term sperm storage. For use in fertilization, sperm must migrate or be moved by the female back down the spermathecal ducts and into the VR (Kotrba 1993).

My dissertation research uses eight laboratory populations of *Teleopsis dalmanni* and two populations of *T. whitei* collected from a variety of geographic locations on the Sunda Shelf region of Southeast Asia. The taxonomic classification of these species has been an issue of recent debate. They were previously referred to as *Cyrtodiopsis dalmanni* and *Cyrtodiopsis whitei*. However, these species were recently reclassified as belonging to the genus *Teleopsis* by Meier and Baker (2002). The phylogenetic relationships among the populations studied here have been examined using 889 bp of two partial mitochondrial gene sequences, cytochrome oxidase II and the 16S ribosomal RNA, and 614 base pairs of one partial nuclear gene sequence, *wingless*. The mitochondrial and nuclear trees are concordant. All but two

pairs of populations (*T. dalmanni* Cameron/Langat and *T. whitei* Gombak/Chiang Mai) form monophyletic groups (Swallow et al. 2005).

Christianson *et al.* (2005) found that all population crosses of *T. dalmanii* that produce offspring have some degree of hybrid sterility or inviability in the lab. This set of populations is ideal for use in examining divergence of reproductive traits and gametic isolation because (1) there is extensive information on their geographic, phylogenetic and reproductive relationships to one another, and (2) the degree of premating and postzygotic isolation are known, and vary as a function of the divergence time between population pairs (Christianson et al. 2005).

Chapter 2: Rapid evolution of reproductive traits among populations of stalk-eyed flies

Abstract

Traits involved in reproduction have a tendency to evolve rapidly via diversifying selection. In stalk-eyed flies, previous studies have shown that both precopulatory and postcopulatory reproductive characters have diverged significantly among species and genera. However, it has not been determined whether such differences exist among populations within species. Divergence in reproductive characters at the intraspecific level is an indicator of the potential for speciation to occur. Here I show that reproductive traits have diverged more rapidly than non-reproductive traits among closely related populations of stalk-eyed flies. I also find evidence of correlated evolution among postcopulatory traits – sperm length and the size of a sperm storage organ – among populations. These results suggest that reproductive traits, and in particular traits involved in postcopulatory interactions, may be undergoing directional selection within populations and have the potential to contribute to reproductive isolation upon secondary contact.

Introduction

Traits involved in male-female interactions have a propensity to diverge rapidly as a consequence of within-population sexual selection (Clark et al. 2007; Gavrilets 2000; Miller and Pitnick 2002). When male-female coevolution is disrupted by population isolation, divergence in courtship rituals, mating preferences of females or the sperm

competitive environment often results (Jennings et al. 2011). Upon secondary contact, such divergence in sexual characters may reduce gene flow between populations and thereby initiate speciation (Mayr 1942). Evidence from a range of taxa variably demonstrates rapid evolution of precopulatory (Grace and Shaw 2011; Seehausen et al. 1997; Shaw 1996; Uy and Borgia 2000), postcopulatory (Minder et al. 2005; Pitnick et al. 2003b), or genitalic (Cordoba-Aguilar 2005; Takami and Sota 2007) morphologies between recently diverged populations. Theory and empirical work demonstrate that such rapid divergence of sexually selected traits can play a causal role in reproductive isolation (Gavrilets 2000; Parker and Partridge 1998; Pitnick et al. 2003a; Rice 1998).

Characters that are exposed to sexual selection are expected to evolve rapidly (West-Eberhard 1983). Divergence in sexually-selected traits among populations can occur as a consequence of genetic drift for female mating preferences via a Fisherian runaway process (Lande 1981). To the extent that features of the female reproductive tract influence fertilization success after mating, a similar process could cause sperm and interacting female traits to diverge in isolated populations. For example, postmating runaway sexual selection has been hypothesized to explain correlated evolution between sperm length and sperm storage size in populations of *Drosophila mojavensis* (Pitnick et al. 2003b).

Stalk-eyed flies (Diptera: Diopsidae) in the genus *Teleopsis* provide an ideal model system in which to assess relative rates of divergence in reproductive and non-reproductive traits, because previous studies and life history characteristics suggest that both pre- and postcopulatory sexual selection should be strong. Stalk-eyed flies

are notable for their precopulatory male ornament, exaggerated eye span, which is under directional sexual selection (Wilkinson and Reillo 1994), is condition-dependent (Cotton et al. 2004; David et al. 2000), and exhibits striking evolutionary lability (Baker and Wilkinson 2001). Eye span is a candidate for involvement in behavioral reproductive isolation, as female preference exhibits correlated change with the male trait (Wilkinson and Reillo 1994). Postcopulatory sexual selection is also expected to be important in stalk-eyed flies because both sexes remate frequently (Wilkinson et al. 2003) with females showing no reduction in receptivity after mating (Grant et al. 2002); females can store sperm from a single mating for up to 30 days (Lorch et al. 1993); and when multiple males mate with a female within a population, paternity can be variable, but on average, is shared equally (Corley et al. 2006; Lorch et al. 1993). Additionally, seminal fluid is known to affect the outcome of sperm competition (Fry and Wilkinson 2004). These conditions indicate a high probability of sperm mixing and the potential for sperm competition, cryptic female choice, and/or postcopulatory sexual conflict to affect reproductive compatibility between populations (Simmons 2001).

Here I measure the rate of divergence in precopulatory, postcopulatory, genitalic, and non-reproductive traits among several pairs of recently diverged allopatric populations of stalk-eyed flies. This approach to evaluating reproductive and non-reproductive trait divergence at the intraspecific level allows me to compare evolutionary rates with phylogenetic replication across classes of morphological characters in the absence of reinforcement and hybridization.

Methods

Fly populations

I used laboratory stocks of six allopatric populations of *Teleopsis dalmanni* and two allopatric populations of *T. whitei* collected from diverse geographic locations (Figure 2-1) on the Sunda Shelf region of Southeast Asia (Swallow et al. 2005). Over a five-year period, from 1996 – 2000, stalk-eyed flies were collected by hand net near streams from nine sites in Thailand, peninsular Malaysia, and the islands of Java, Sumatra, and Borneo. A hypothesis of the phylogenetic relationships among these populations is shown in Figure 2-2 (Swallow et al. 2005). Since the time of collection, laboratory populations have been maintained in the lab in large cages at 25^{o}C and 70% relative humidity on a 12L:12D following standard procedures (Lorch et al. 1993). The experiments described here were conducted in 2004-2005.

Rearing conditions

Upon eclosion, flies were stored in small (16 x 14 x 12.5 cm) plastic cages. While external morphological traits in holometabolous insects are fixed in size after the adult cuticle hardens, internal reproductive traits could change after eclosion. For example, testis and accessory gland (AG) size are age-dependent in stalk-eyed flies (Baker et al. 2003). Therefore, I controlled for age by dissecting flies every 2-3 days between 14 and 40 days after eclosion. In total, I reared, dissected and measured 383 flies, which included at least 16 individuals per sex per population.

Dissection and trait measurements

I measured a suite of male and female reproductive traits, which I chose on the basis of their likelihood to experience precopulatory and postcopulatory sexual selection (Kotrba 1993; Lorch et al. 1993) or are required for successful copulation. To compare traits involved in different reproductive functions, I assigned each trait to one of four trait types: precopulatory, genitalic, postcopulatory, and non-reproductive. I measured three internal male postcopulatory traits: accessory gland (AG) area, testis area and sperm length. Additionally, I measured the area, length, and width of three male genitalic traits (Figure 2-3): the ejaculatory apodeme, the aedeagal apodeme, and the surstyli. I also measured seven female postcopulatory traits (Figure 2-4): spermathecal area, spermathecal duct length, accessory gland area, ventral receptacle (VR) length, size of VR chamber, number of VR chambers, and average length of three mature eggs. I considered male eye span to be a precopulatory trait and wing area, wing length, and tibia length were measured as non-reproductive traits. I used body length to remove effects of size from all traits (see Statistical Analysis).

I anesthetized each fly with carbon dioxide, removed the abdomen and placed the remainder of the body in an eppendorf tube, which was frozen at -20 °C for later measurement of external morphological traits. The abdomen was then placed on a glass slide and the reproductive tract dissected into 40µl of phosphate-buffered saline (PBS). Traits were visualized on a Nikon Eclipse E600 compound microscope with a Cohu CCD camera. Video images were digitized with a Macintosh computer and traits were measured using NIH image v.1.62.

In males, the area of the testes and the accessory glands were measured at 100X. After measurement, testes were punctured at the proximal end with forceps and swirled in PBS to release mature sperm bundles. A cover slip was then applied and the length of five randomly chosen, intact mature sperm bundles was measured at 400X using Nomarski illumination. In females, the reproductive tract was oriented with the ventral sclerite facing up, and a coverslip was gently placed over the entire reproductive tract with the exception of the eggs. Eggs were counted and the length of three mature eggs (when available) measured at 100X. All other female reproductive tract traits were measured at 400X.

To isolate the external, sclerotized portions of the male genitalia, the terminal segment of the abdomen was placed in a 1.5 ml tube with two drops of NaOH and dropped in a beaker of boiling water for 60 seconds to dissolve soft tissue. The aedeagal apodeme, ejaculatory apodeme, and surstyli were then separated on a glass slide in a drop of glycerol and measured at 200X after applying a cover slip.

To measure external morphological traits, flies were oriented so that the body was balanced on the thoracic spines, and eye span, body width and body length were measured at 11X using NIH Image. Eye span was recorded as the distance between the outer edges of the ommatidia, body width as the widest part of the thorax, and body length from face to wing tip. Wings and tibia were removed from the body with forceps and mounted on a glass slide. They were then measured at 11X using NIH Image.

Statistical Analysis

Effects of age, body size and population

Statistical analyses were performed using JMP v.5.0.1 (SAS Institute). To determine whether reproductive traits differ among allopatric populations, I performed analyses of covariance (ANCOVA) using population nested within species and species as random effects, and body length and age as covariates. Because I conducted 21 separate ANCOVAs, I applied a sequential Bonferroni procedure (Rice 1989) to assign significance. To remove covariate effects on trait values I used least squared means (LSMs) from the ANCOVA in all subsequent analyses.

To reduce collinearity among traits, I calculated pairwise correlations between traits likely to be correlated (for example, multiple measures of the same trait) using LSM values. If an r^2 value of greater than 0.1 occurred among such measurements, the trait that was least correlated with other characters was kept in the analysis. For example, the length, area and width of the ejaculatory apodeme were all highly correlated. For this trait I analyzed only the rate of divergence in width because it was least correlated with aedeagal apodeme and surstylus size (the other male genitalia characters that were measured). I performed the same procedure on the non-reproductive characters and found significant correlations between wing area and both wing length and tibia length. Therefore, male and female wing areas were excluded from subsequent analyses. Wing length was also excluded from the evolutionary rate analyses because it was highly correlated with both tibia length and body length.

Evolutionary rate

In order to assess whether the morphological characters measured here have diverged among closely related populations, I calculated the evolutionary rate of change for each trait. To calculate rates of evolution, I used the four most recently diverged pairs of populations: *T. dalmanni* Cameron/Langat, Soraya/Bukit Lawang, Gombak/Bukit Ringit and *T. whitei* Gombak/Chiang Mai (cf. figure 2-2). All but one of these population pairs (Langat/Cameron) exhibit reciprocal monophyly for two mitochondrial genes (Swallow et al. 2005). Lack of reciprocal monophyly between populations could be due either to recent divergence or gene flow, both of which should impair detection of trait differences between populations. Therefore, the inclusion of the Langat/Cameron population pair in spite of reciprocal monophyly makes my estimate of rates of evolution more conservative. Indeed, this method for calculating rate of change results in a minimum estimate of the evolutionary rate for any given character as homoplasy or fluctuating selection will not be detected.

Rate of divergence was calculated using the following formula, which is similar to Haldanes (Gingerich 1993).

$$\text{Evolutionary rate} = \left| \frac{\left(\frac{X_2}{S_p}\right) - \left(\frac{X_1}{S_p}\right)}{\%\ BP\ Divergence} \right|$$

where X_1 and X_2 represent the trait means for populations 1 and 2 and S_P is the pooled standard deviation for the trait. Percent basepair difference between populations was calculated using data from two mitochondrial gene fragments, COII and 16S (Swallow 2005). Time was assumed to be proportional to the genetic distance between each population pair, which was estimated by averaging genetic distance among pairs of individuals using Jukes-Cantor distances in PAUP*v.4.0b10 (Swofford 2003).

To determine if evolutionary rate differed among trait types (precopulatory, postcopulatory, genitalic, and non-reproductive), I calculated average least square mean rates for each trait across population nodes, and then compared rates by ANOVA which included sex and trait type as factors.

Correlated evolution

In order to determine if male and female postcopulatory trait values exhibit correlated evolution among populations, I computed phylogenetically independent contrasts (Felsenstein 1985) using CAIC v.2.6.9 (Purvis and Rambaut 1995). Independent contrasts control for statistical non-independence caused by common ancestry by using differences in trait values between taxa rather than trait means of species (Harvey and Pagel 1991). I used the phylogenetic hypothesis proposed by Swallow et al. (2005) to test for coevolution of sperm length with two female reproductive tract characters, spermathecal area and VR size. These are the same traits that Presgraves *et al.* (1999) found to be significantly correlated across species and genera of stalk-eyed flies. I performed least-squares regression analysis forced through the origin on independent contrast values to test whether the traits exhibit correlated change (Harvey and Pagel 1991).

Results

Effects of age, body size and population

The degree to which reproductive and non-reproductive traits differed as a function of age, body length, population, and species is summarized in Table 2-1. All external

morphological traits covaried with body length, but only the sexually selected precopulatory trait eye span differed among populations. In contrast, none of the male genitalia traits covaried with body size. Only aedeagal apodeme length changed with age and only surstylus width and length differed among populations, although all male genitalic traits differed between the two species with the exception of aedeagal apodeme length. Of the internal reproductive traits measured in males, only sperm length differed among populations and did not covary with age or body size. In contrast, both testis size and accessory gland size covaried strongly with age but neither differed among populations. Testis size, but not accessory gland size, also covaried with body size.

Female non-reproductive traits covaried with body size similarly to males. Interestingly, no female traits covaried with age. Of the female internal traits, only spermathecal area and spermathecal duct length covaried with body size. All internal female traits exhibited highly significant differences among populations.

Evolutionary rate

An ANOVA on evolutionary rate by trait category (precopulatory, genitalic, postcopulatory and non-reproductive) revealed no effect of sex ($F_{1,3} = 1.56$, $P = 0.22$) but a significant effect of trait type on rate of evolution ($F_{4,3} = 7.20$, $P = 0.0012$). Post-hoc Tukey's HSD T tests (Figure 2-5) indicate that precopulatory and postcopulatory trait rates do not differ from each other and both are evolving faster than genitalia and nonreproductive traits, which also do not differ.

Correlated evolution

Regression analysis of male and female reproductive traits using independent contrasts revealed a positive trend between sperm length and size of the VR chamber (slope = 0.01, t = 2.25, $r^2 = 0.39$; p = 0.055). In contrast, change in sperm length was not correlated with change in spermathecal area (slope = -20.95, t =-1.2, $r^2 = 0.15$, p = 0.26).

Discussion

I find evidence of significant diversification in many reproductive traits among allopatric populations of *Teleopsis* stalk-eyed flies. Sperm length, several measures of the male genitalia, eye-span, and many metrics of the female sperm storage organs are all significantly different among populations within species (table 2-1). Additionally, the evolutionary rate analysis demonstrates that among the most closely related populations, the precopulatory male ornament and postcopulatory traits have diverged more rapidly than non-reproductive traits. Rapid divergence of sexually selected traits among closely related populations is consistent with diversification driven by sexual selection acting on these characters within allopatric populations. Christianson ***et al.*** (2005) found evidence for premating reproductive isolation among some of these populations – indicating that the differences in male eyespan observed here may play a role in premating isolation among populations. I therefore focus discussion here on the divergence in postcopulatory traits, as it has not yet been determined whether

postcopulatory prezygotic (gametic) reproductive isolation exists among these populations of stalk-eyed flies.

I found evidence of significant divergence in several postcopulatory traits. Specifically, sperm length and the size of the female sperm storage organs are significantly different among populations within species. Sperm size and female reproductive tract morphology are known to coevolve within populations as a result of postcopulatory sexual selection (Jennings et al. 2011). Stochastic divergence among allopatric populations as a result of within-population postcopulatory sexual selection is likely to lead to diversification in morphometry of traits associated with sperm competition and cryptic female choice. Such diversification has significance for potential speciation (Coyne and Orr 2004)

However, postcopulatory trait differences among populations need not necessarily be caused by sexual selection. Several hypotheses can explain faster divergence of postcopulatory reproductive traits than non-reproductive traits among closely related populations: (1) selection among trait types is similar, but postcopulatory traits have higher genetic variances than non-reproductive trait types; (2) postcopulatory reproductive traits are subject to genetic drift while all non-reproductive traits are under stabilizing selection; or (3) diversifying selection acts more strongly on postcopulatory traits than on non-reproductive traits.

The first hypothesis seems unlikely given that sperm length is heritable (Johns and Wilkinson 2007) and female storage organ sizes exhibit correlated change with eye span (Wilkinson et al. 2005). No evidence suggests that the magnitude of genetic variation for these postcopulatory traits is exceptionally high. Genetic drift could

explain rapid divergence when populations are small and traits are unrelated to fitness. However, given the importance of successful fertilization to fitness, the observed pattern of repeated rapid evolution, and amount of genetic variation segregating within populations (Swallow et al. 2005), drift also seems unlikely to be solely responsible for the observed patterns (Coyne and Orr 2004). Moreover, strong stabilizing selection on non-reproductive traits would result in less variability for slowly evolving than for rapidly evolving traits. However, coefficients of variation do not differ among trait types ($F = 1.52$, $P = 0.24$, ANOVA) and do not correlate with rate of evolution ($r^2 = 0.0003$; $P = 0.94$). Genetic drift could, however, have contributed to the initial diversification of postcopulatory traits upon the founding of new populations. Such events seem likely to have occurred repeatedly in the past given that the Sunda Shelf region has frequently experienced major volcanic events and oceanic incursions during glacial minima (Swallow et al. 2005). Founder events may, therefore, have allowed postcopulatory reproductive traits to take divergent selective trajectories.

Evidence in support of the diversifying selection hypothesis comes from previous studies (Miller and Pitnick 2002; Ramm et al. 2009; Simmons 2001; Snook et al. 2009), theoretical predictions (Gavrilets 2000; Gavrilets and Waxman 2002) and patterns of correlated change between male and female postcopulatory traits (Anderson et al. 2006; Jennings et al. 2011; Pitnick et al. 1999; Presgraves et al. 1999). In *Teleopsis* stalk-eyed flies, the VR is made up of approximately 50 chambers, each capable of storing a single, coiled sperm prior to fertilization (Kotrba 1993). I found that change in VR chamber size tended to correlate with change in sperm length across populations, which is consistent with functional interaction between these traits

given that they are not genetically correlated (Wilkinson et al. 2005). Additionally, Presgraves *et al.* (1999) found evidence for correlated evolution between sperm length and both spermathecal area and VR size across species of stalk-eyed flies, a pattern that has also been observed among populations within species in other taxa (Michalczyk et al. 2011; Minder et al. 2005; Pitnick et al. 2003b; Ronn et al. 2011; Sanchez et al. 2011). These results are consistent with rapid evolution of postcopulatory morphologies driven by coevolution between male and female traits.

An additional factor that may contribute to divergence in postcopulatory characters across populations of diopsid flies is that divergence in sex ratio among populations, mediated by X-chromosome meiotic drive, may have altered the sperm competitive environment between populations and therefore changed the intensity of postcopulatory sexual selection. X-chromosome drive is present in every population used in this study and the frequency of multiple mating is concordant with the frequency of drive in different species (Wilkinson et al. 2003). Females are expected to remate more often when drive is common to increase the chance that they will mate with a non-drive male. Fry and Wilkinson (2004) found that non-drive males have a postcopulatory competitive advantage when competing for fertilizations with males carrying the driving X.

Characters that have diverged most since lineage splitting are expected to contribute to reproductive isolation upon secondary contact (Coyne and Orr 2004; Mayr 1942). The capacity of postcopulatory sexual selection to create reproductive isolating barriers has only been considered recently (Eady 2001; Howard 1999; Snook et al. 2009). Due to their rapid and correlated divergence, sperm and sperm storage

organ morphometry are strong candidates for involvement in the evolution of reproductive isolation in diopsid stalk-eyed flies and other taxa. Variation in sperm length and sperm storage organ morphology has been found to result in differential fertilization success in *Drosophila* and in a field cricket (Garcia-Gonzalez and Simmons 2007a; Miller and Pitnick 2002). My results demonstrate that taxa with exaggerated precopulatory ornaments may still experience strong postcopulatory sexual selection, and therefore divergence in postcopulatory traits may drive reproductive isolation in such systems. Further studies that directly address the relationship between rapid evolutionary change and reproductive isolation will enhance our understanding of the functional implications of rapid divergence in postcopulatory characters.

Tables

Table 2-1. F-ratios from nested ANCOVAs testing for effects of population nested within species and species with age and body length as covariates on male and female morphological traits in stalk-eyed flies. Significance values shown reflect adjustment of alpha using the sequential Bonferroni procedure. * P < 0.05; **P<0.001; *** *P* < 0.0001

trait	*trait category*	*age*	*body length*	*population (species)*	*species*
male traits					
testis area	postcop	33.7***	20.7***	2.6	13.1*
sperm length	postcop	0.9	0.0	24.0***	34.5*
accessory gland area	postcop	60.7***	10.5	2.4	3.3
surstylus width	genitalic	0.2	6.8	7.5***	447.1***
surstylus length	genitalic	0.0	5.7	252.3***	95.4***
ejaculatory apodeme width	genitalic	5.1	0.1	2.0	15.3*
aedeagal apodeme width	genitalic	3.5	0.7	2.8	18.8*
aedeagal apodeme length	genitalic	12.8**	0.8	1.8	5.3
eye span	precop	6.4	255.7***	53.1***	0.0
body width	somatic	0.4	127.9***	3.5*	30.7*
tibia length	somatic	3.0	14.2**	1.7	0.2

trait	*trait category*	*age*	*body length*	*population (species)*	*species*
female traits					
spermathecal area	postcop	5.0	33.5***	100.0***	3.1
spermathecal duct length	postcop	2.9	13.2**	19.2***	1.9
VR length	postcop	1.3	7.9	62.5***	0.3
VR chamber size	postcop	2.5	1.1	14.9***	2.7
accessory gland area	postcop	0.8	8.2	11.9***	0.1
egg size	postcop	1.3	1.4	4.3**	2.4
eye span	precop	0.1	255.8***	37.4***	0.9
body width	somatic	0.4	68.2***	6.3***	28.5*
tibia length	somatic	0.7	10.9*	0.6	0.1

Table 2-2. Mean values of each trait used in the analyses, broken down by species name (top row) and column name (second row). The first table shows male traits and the second shows female traits.

	Teleopsis dalmanni						*Teleopsis whitei*	
	Gombak	Bukit Ringit	Bukit Lawang	Soraya	Cameron	Langat	Gombak	Chaing Mai
male traits								
testis area (mm^2)	0.574	0.667	0.714	0.553	0.646	0.650	0.525	0.534
sperm length (μm)	169.9	174.2	167.2	187.1	174.0	165.2	194.3	187.5
accessory gland area (mm^2)	0.150	0.111	0.124	0.106	0.098	0.135	0.082	0.104
surstylus width (μm)	68.7	70.0	71.4	75.2	82.3	80.1	39.0	39.7
surstylus length (μm)	209.7	207.9	203.4	212.8	150.1	132.4	167.2	165.6
ejaculatory apodeme width (μm)	230.5	217.2	241.9	218.5	204.6	247.5	184.1	159.1
aedeagal apodeme width (μm)	112.8	105.4	115.3	105.9	116.1	125.9	139.6	166.7
aedeagal apodeme length (μm)	301.5	308.2	336.1	323.0	358.4	359.6	314.4	277.7
eye span (mm)	7.76	8.62	8.68	8.43	9.43	9.43	9.51	8.27
body width (mm)	1.75	1.80	1.84	1.66	1.90	1.89	1.72	1.61
tibia length (mm)	1.62	1.77	1.84	1.72	1.87	1.86	1.82	1.72

	Teleopsis dalmanni						*Teleopsis whitei*	
	Gombak	Bukit Ringit	Bukit Lawang	Soraya	Cameron	Langat	Gombak	Chaing Mai
female traits								
spermathecal area (μm^2)	2698.9	3115.0	3090.7	2415.5	3886.1	4647.2	2560.4	2038.7
spermathecal duct (μm)	366.5	377.1	370.4	385.0	308.4	384.1	390.4	401.4
accessory gland area (μm^2)	6257.1	4509.8	3705.5	4055.9	5819.4	5814.2	5847.7	4566.6
VR length (μm)	65.1	75.4	73.8	80.3	88.0	85.0	78.1	84.7
VR chamber size (μm)	2.43	2.09	2.00	1.92	2.21	2.26	2.34	2.44
egg size (mm)	0.785	0.763	0.801	0.772	0.800	0.800	0.772	0.772
eye span (mm)	5.43	5.87	5.78	5.64	6.21	6.34	6.01	5.44
body width (mm)	1.73	1.77	1.73	1.65	1.83	1.88	1.60	1.47
tibia length (mm)	1.44	1.45	1.42	1.33	1.45	1.48	1.45	1.37

Figures

Figure 2-1. Map of population collection sites in South East Asia. Adapted from Swallow *et al* (2005)

Figure 2-2. The phylogenetic relationships among the populations used in this study were hypothesized by maximum parsimony from Swallow et al. (2005) using 889 base pairs of two mitochondrial gene sequences, cytochrome oxidase II and the 16S ribosomal RNA, and 614 base pairs from a nuclear gene, wingless. The mitochondrial and nuclear trees are concordant and all but one pair of populations (*T. dalmanni* Cameron/Langat) show conclusive reciprocal monophyly (Swallow et al. 2005).

Figure 2-3. Images of the three aspects of male genitalia measured in this study, demonstrating the differences between T. whitei (left column) and T. dalmanni (right column). (a) and (b) are ejaculatory apodemes, (c) and (d) are aedeagal apodemes and (e) and (f) are the surstyli (graspers). Aedeagal apodeme length was measured across the top of the organ based on landmarks consistent across all species. Surstylus width was measured at the narrowest point.

Figure 2-4. Image of the female reproductive tract. Lines indicate how measurements were taken: (a) spermathecal area, (b) spermathecal duct length, (c) accessory gland (ag) area, (d) ventral receptacle (vr) length. All three spermathecal heads, both spermathecal ducts, and both accessory glands were measured and the average for each female was used in all analyses.

Figure 2-5. Results of an ANOVA comparing the evolutionary rates of divergence in precopulatory, postcopulatory, genitalic and non-reproductive traits among closely related populations ($F_{4,3} = 7.20$, $P = 0.0012$.)

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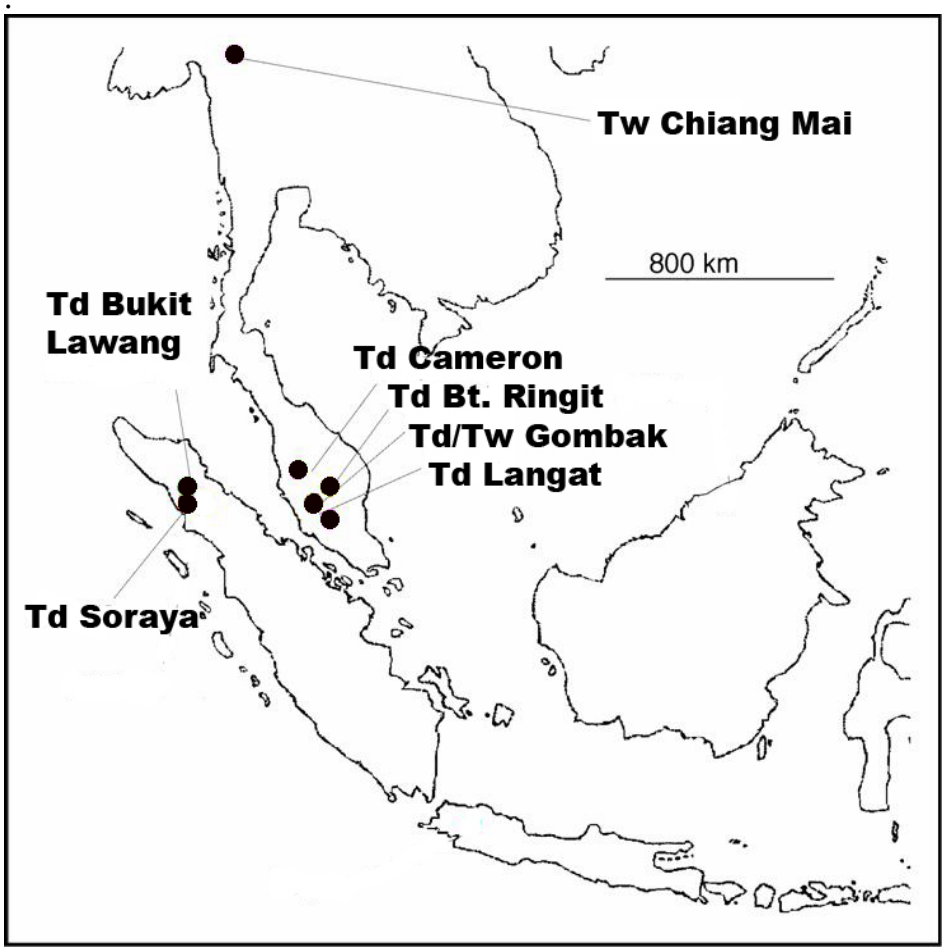

Tw Chiang Mai
800 km
Td Bukit
Lawang
Td Cameron
Td Bt. Ringit
Td/Tw Gombak
Td Langat
Td Soraya

Figure 2-1

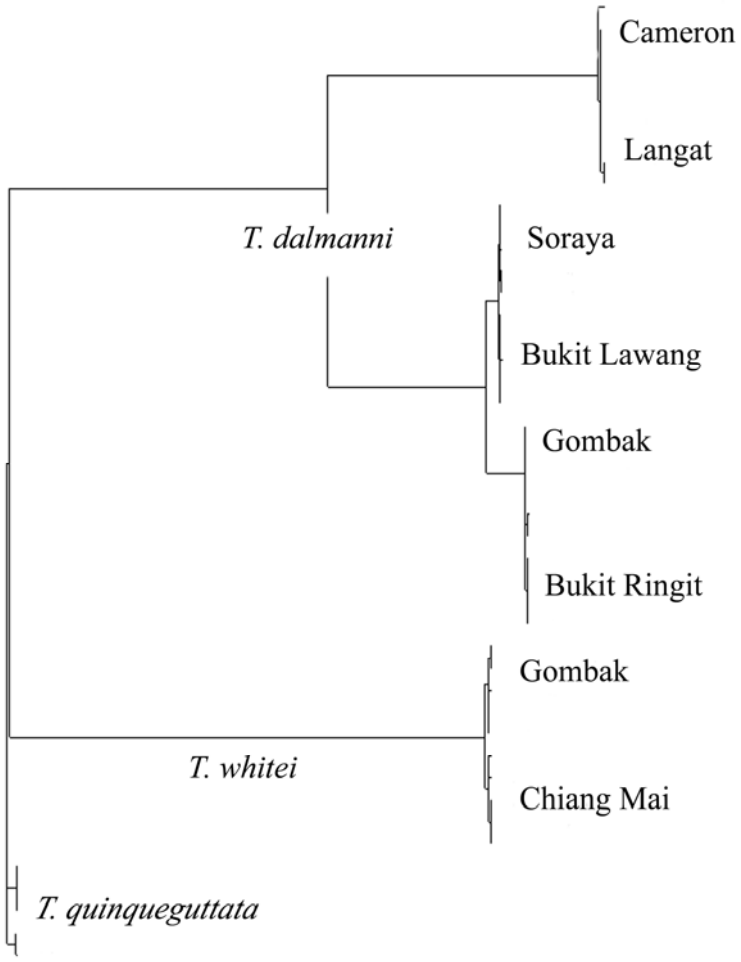

Cameron
Langat
T. dalmanni
Soraya
Bukit Lawang
Gombak
Bukit Ringit
Gombak
T. whitei
Chiang Mai
T. quinqueguttata

Figure 2-2

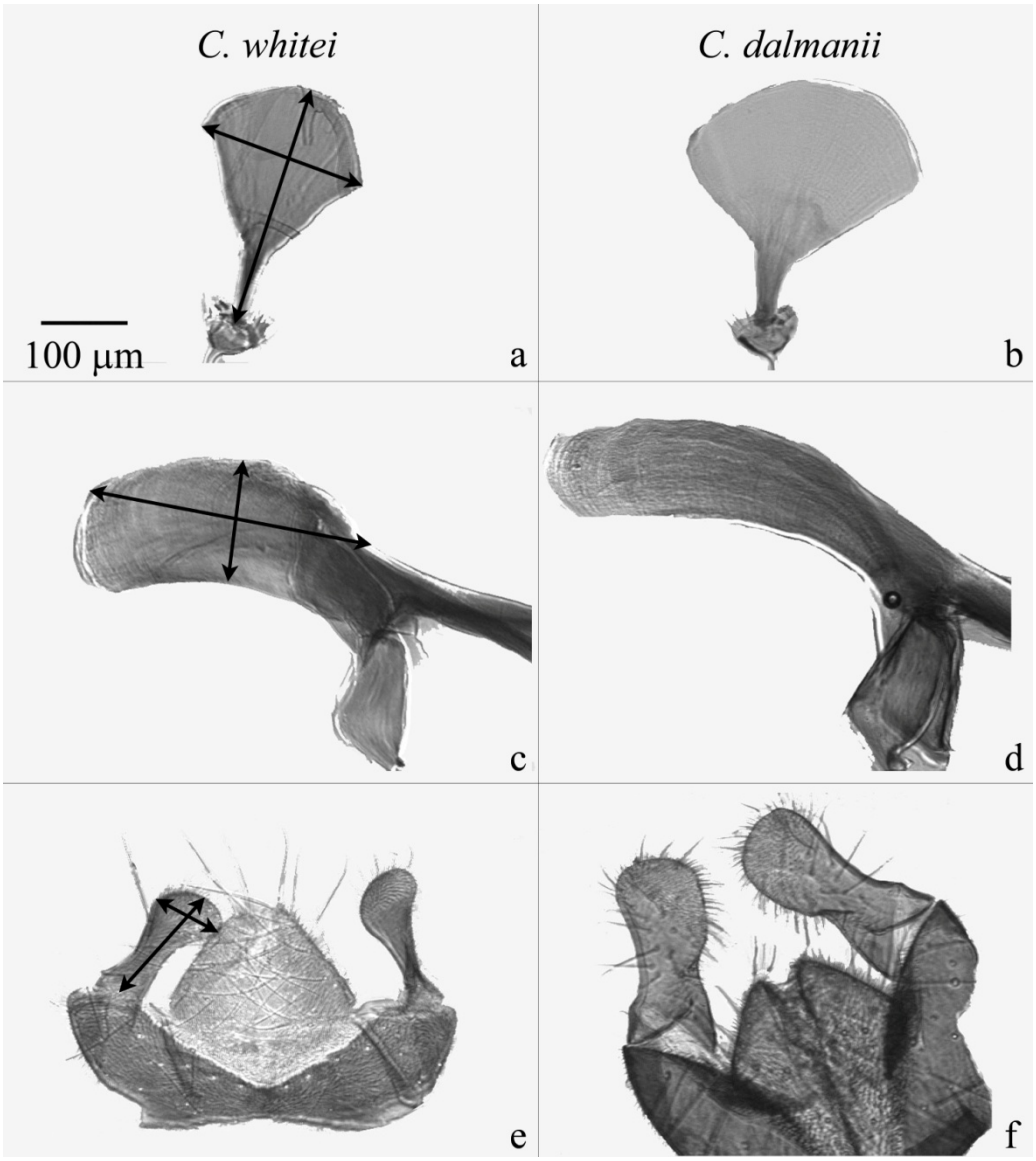


Figure 2-3

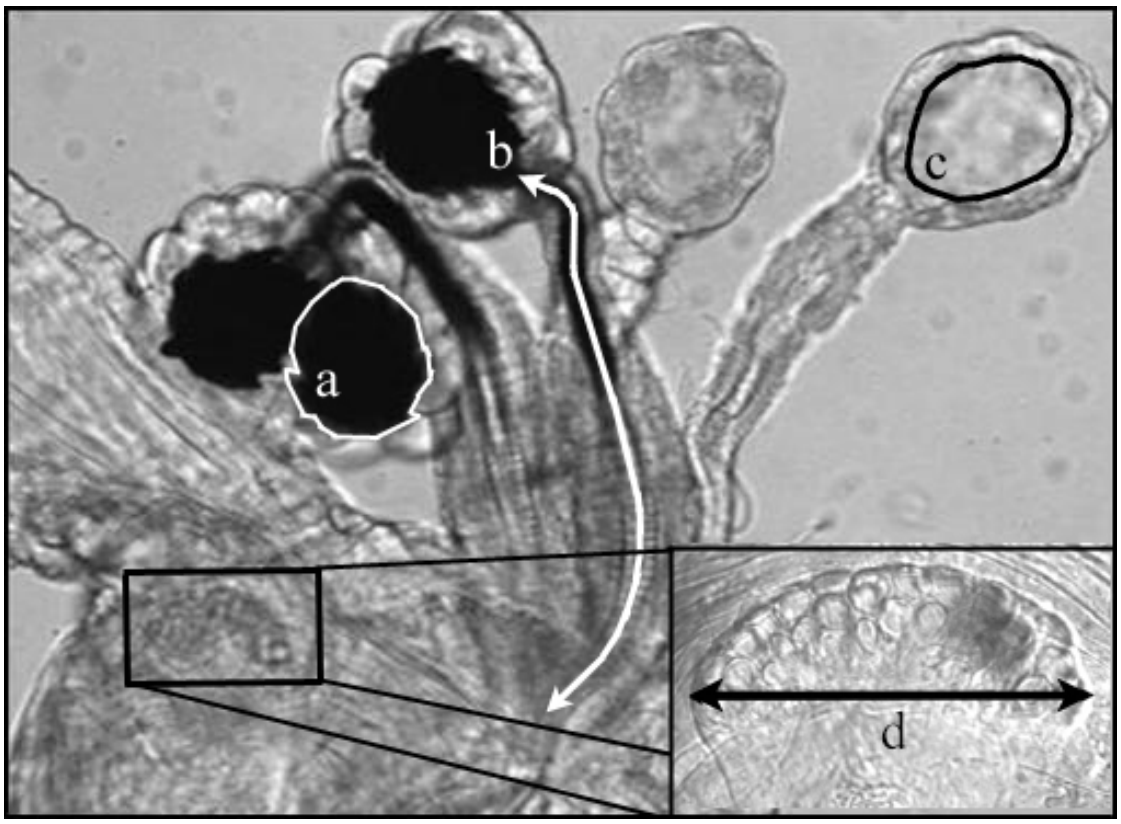


Figure 2-4

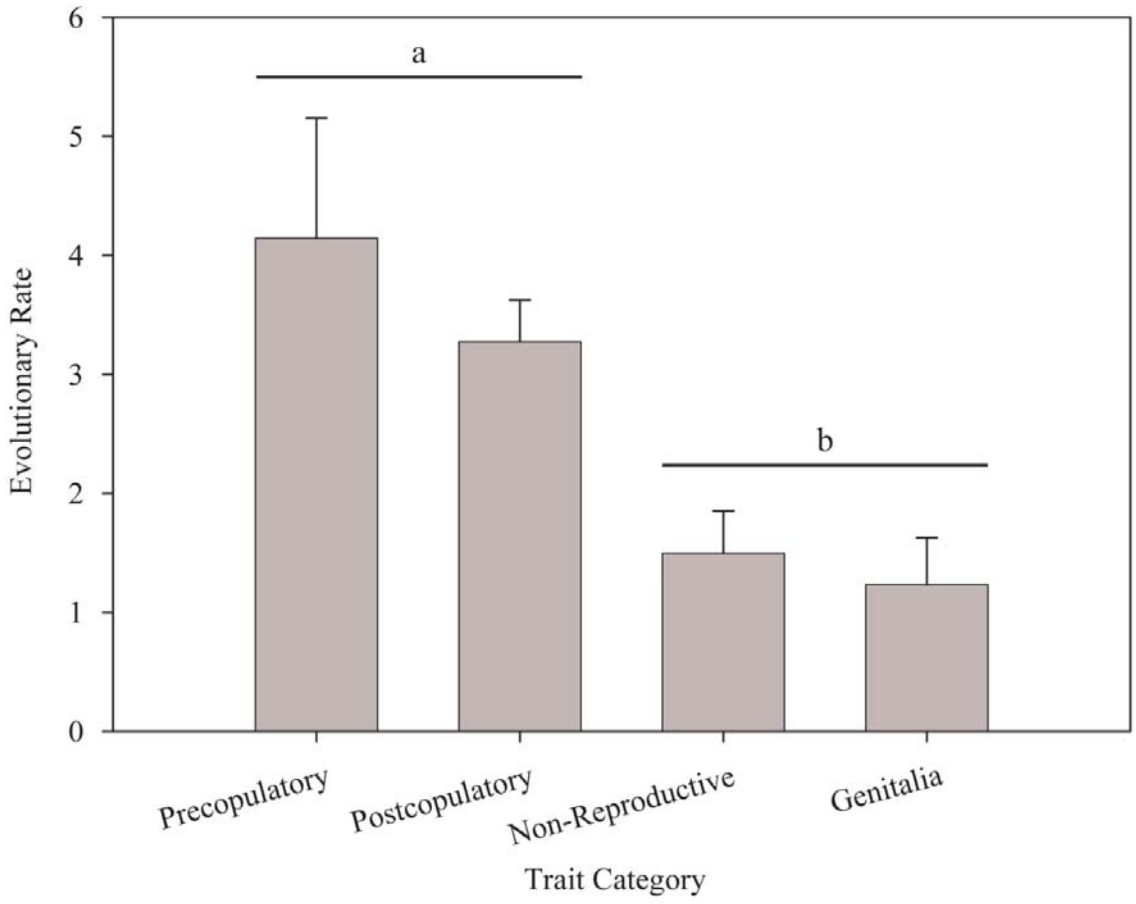

6
5
4
3
2
1
0
Evolutionary Rate
a
b
Precopulatory
Postcopulatory
Non-Reproductive
Genitalia
Trait Category

Figure 2-5

Chapter 3: Mechanisms of non-competitive gametic isolation in stalk-eyed flies

Abstract

Postcopulatory sexual selection is a strong evolutionary force known to affect within-population evolutionary dynamics of multiple traits in a wide variety of taxa. However, the ability of postcopulatory sexual selection to contribute to reproductive isolation has only recently been considered. This form of reproductive isolation, termed gametic isolation, involves a breakdown in cross-population compatibility at any stage between copulation and fertilization. Here, I present a comprehensive analysis of non-competitive gametic isolation – barriers that occur in the absence of sperm competition – between four populations of stalk-eyed flies (*Teleopsis*). First, I distinguish between gametic isolation and postzygotic isolation by assessing between-population fertilization and hatching success. I find that the majority of unhatched eggs between populations failed to hatch because they were not fertilized, not because of embryonic inviability, indicating that gametic isolation exists among these populations. I then measure six possible mechanisms of gametic isolation in order to identify the reason for reproductive breakdown between copulation and fertilization. I find that an important mechanism of gametic isolation in *Teleopsis* is the ability of sperm to reach the site of fertilization; sperm are significantly less successful at reaching the site of fertilization in heteropopulation crosses, which leads to a decrease in fertilization success.

Introduction

Historically, reproductive isolating mechanisms have been categorized as acting either before or after zygote formation. Until recently, the majority of research on prezygotic isolation has focused on behavioral isolation at the time of mating and thus has failed to assess the potential importance of postmating prezygotic isolation, also called gametic isolation (Coyne and Orr 2004; Eady 2001; Howard 1999; Markow 1997). Empirical work examining the effect of divergence in fertilization systems on reproductive incompatibilities has only recently emerged. Of those studies which have examined gametic isolation, only a few (Alipaz et al. 2001; Brown and Eady 2001; Price et al. 2001) include more than two species or have been conducted at the intraspecific level.

Over the past several decades, research and theory on sexual selection has been expanded to include events that occur after copulation but before fertilization and involve interactions between sperm or seminal products produced by males and females (Eberhard 1996; Parker 1970; Simmons 2001). More recently, the potential for these postcopulatory interactions to create barriers to gene flow among populations has gained interest (Eady 2001; Howard 1999; Ludlow and Magurran 2006; Martin-Coello et al. 2009). Coyne and Orr (2004) divide gametic isolating barriers into two forms: non-competitive and competitive. Non-competitive gametic isolation impedes fertilization between populations regardless of whether the female has mated with one or multiple males. Such incompatibilities among fertilization systems can arise at any stage between copulation and fertilization, and are likely to evolve as a byproduct of rapid, postcopulatory sexual selection occurring within populations (Coyne and Orr

2004; Price et al. 2001). Competitive gametic isolation occurs when the conspecific male out-competes the heterospecific male during sperm competition. In the current study, I investigate only non-competitive gametic isolating barriers.

Using diopsid stalk-eyed flies in the genus *Teleopsis*, Christianson *et al.* (2005) found that postzygotic reproductive isolation, in the form of male hybrid sterility, could be detected in any population cross that resulted in offspring. They also reported that progeny production decreased in population crosses as a function of genetic distance, but could not discriminate whether this effect was due to gametic isolation or postzygotic isolation (i.e. embryonic inviability). Here I distinguish between these two possibilities by determining the proportion of eggs that hatch between populations and by further examining whether unhatched eggs are fertilized or not. In doing so I am able to determine whether the decrease in progeny production among populations observed by Christianson *et al.* (2005) is due to non-competitive gametic isolation or hybrid inviability.

Incompatibility between the fertilization systems of allopatric populations or species have been found to occur at any stage of the process between copulation and fertilization. For example, Price *et al.* (2001) found evidence of decreased sperm transfer and decreased sperm storage in hybridizations within the *Drosophila simulans* species complex. Dean and Nachman (2009) showed that heterospecific males are slower to fertilize eggs than conspecific males in crosses between two species of house mice, *Mus domesticus* and *M. musculus*. Other known mechansisms of gametic isolation include decreased oviposition (Brown and Eady 2001), incomplete fertilization (Alipaz et al. 2001), and sperm competitive disadvantage (Gregory and

Howard 1994; Howard 1999). Here I examine several possible mechanisms of gametic isolation in diopsid stalk-eyed flies.

To determine whether non-competitive gametic isolation is occurring in stalk-eyed flies, and how the processes leading to fertilization have been affected by change over evolutionary time, I use four populations of *Teleopsis dalmanni* which span a range of genetic distances and exhibit reciprocal monophyly (Swallow et al. 2005). I conduct a series of crosses within and between these populations to collect data on sperm transfer, sperm survival, sperm motility, sperm storage, sperm movement to the site of fertilization, egg fertilization and egg hatch. By comparing results from crosses between populations (heteropopulation) to within populations (conpopulation) I assess both the presence and magnitude of gametic incompatibility. I then use these results to identify general patterns regarding which barriers to reproduction are important in this system and at what stage of genetic divergence those reproductive isolating barriers arise.

Methods

Population samples

Flies were collected from sites on the major land masses in the Sunda shelf region of South-East Asia. This area covers the known range of the most well studied *Teleopsis* species, *T. dalmanni*, which was synonymized with *Cyrtodiopsis dalmanni* (Meier and Baker 2002). A recent paper has suggested reversion to *Cyrtodiopsis* {Feijen, 2011 #1599}, however, I have decided to follow Meier and Baker (2002) and use the *Teleopsis* genus name here. Over a five year period, stalk-eyed flies were collected by

hand net near streams from nine sites in Thailand, peninsular Malaysia, and the islands of Java, Sumatra, and Borneo (Swallow et al. 2005). Flies used in this study were from populations collected 3-7 years before the experiments were conducted. Populations have been maintained in the laboratory in large plexiglass cages.

Rearing Conditions

To collect flies for mating, three 150ml cups containing 75 ml of pureed corn (containing 0.5% methylparaben to inhibit mold growth) were placed in population source cages for 3 or 4 days. These cups were then removed from the cage and placed in a larger container lined with damp cotton and plugged with a foam stopper to permit the larvae to climb out of the cup to pupate. Flies were reared at 25 ^{o}C on a 12L:12D cycle. Upon eclosion, flies were collected and placed in a small cage (16 x 14 x 12.5 cm) with moist cotton and blotting paper on the bottom to enhance humidity. Within three days of eclosion males and females were placed in separate cages to ensure that all flies used in experimental crosses were virgins, as sexual maturity occurs between two and three weeks after eclosion (Baker et al. 2003). All cages were fed pureed corn in disposable dishes twice a week unless otherwise indicated.

Mating protocol

Two sets of mating crosses were performed. In the first set of crosses, I measured egg hatch, sperm number and sperm survival. In the second set of crosses, I determined fertilization success, sperm motility and ability of sperm to reach the site of fertilization. The use of two sets of crosses was necessitated by the fact that assessing

fertilization success arrests egg development; consequently, hatch success and fertilization success cannot be measured from the same crosses.

I chose populations based on their genetic similarity (Swallow et al 2005) and evidence indicating that some amount of postmating reproductive isolation is present (Christianson et al. 2005). I attempted, when possible, to conduct replicate crosses between independent populations with similar genetic distances. I crossed four populations of *T. dalmanni* originating from Gombak, Bukit Lawang, Soraya, and Cameron (see map, chapter 2). The populations were crossed in a full factorial design. A total of 275 crosses were conducted, with an average of 17 replicates per population pair.

For both sets of crosses, three virgin females and one virgin male of the appropriate populations were placed in a small cage (16 x 14 x 12.5 cm) and allowed to copulate freely for seven days. To minimize possible age-related effects on fecundity I used individuals between 6 and 12 weeks of age. This age span ensures that all flies are sexually mature (Baker et al. 2003).

After seven days, a folded piece of black construction paper soaked in a corn/water mixture was placed in a weigh boat in the cage as the site for oviposition. Black construction paper was used for contrast to facilitate egg collection. This paper and one weigh boat containing instant *Drosophila* food (Wards Scientific), which the flies will eat but not use for oviposition, were placed in the cage for three days. In the first set of crosses, the paper was subsequently placed in a plastic container with damp cotton for one week. There, eggs were allowed to hatch. Hatching success was determined as described below. For the second set of crosses, the paper was placed in

the same plastic container and set aside for three days after removal from the cage to allow fertilized eggs to develop. Fertilization success was then measured as described below.

Hatch Success

To quantify hatching success, eggs were counted directly on the construction paper through a dissecting microscope by prodding each egg with a fine probe one weeks after removal from the cage. Hatched eggs had a distinct appearance – only an empty chorion remained on the paper and it deflated upon probing. Eggs that had not hatched remained intact upon probing and either had a solid or opaque appearance – possibly due to lack of fertilization or development. Both types of eggs were scored as unhatched. The number of hatched and unhatched eggs was tallied for each cross.

Fertilization Success

In order to assess whether unhatched eggs were fertilized, all eggs were counted and assessed for hatching success as described above. Unhatched eggs were then counted and collected. To evaluate fertilization success, I removed the chorion and the vitelline membrane. Eggs were then stained and examined under UV to assess whether cellular division had occurred.

Details of this procedure are as follows. Unhatched eggs were placed in a mesh basket constructed from a plastic scintillation vial. The vial was cut in half with a hole cut in the lid and fine Nytex mesh placed over it. The chorion was removed from the eggs by two minutes of immersion in 50% commercial bleach with intermittent

stirring. Eggs were then transferred to a glass vial for removal of the vitelline membrane. I followed the protocol of Weischaus and Nusslein (1986) with the following modifications. First, 1.5 ml water, 2.5 ml heptane, and 225 µl each of formaldehyde, phosphate buffer, and potassium manganese were added to the vial. The vial was then vortexed for one minute. After vortexing, the solution settled into two layers and the lower layer was removed and discarded. Two ml of methanol were added and the vial was vortexed for one minute again. Once the solution settled, the top layer was removed and another 1 ml of methanol was added. The vial was inverted several times and all liquid was removed and discarded. Eggs were then transferred from the vial and placed in a drop of phosphate buffered saline (PBS) on a glass slide. Cells were stained by adding one drop of 10^{-7} Hoechst 33258 and then examined at 100X with UV fluorescence using a Nikon Eclipse E600 microscope. Eggs in which multiple cell nuclei were observed were scored as fertilized. Figure 3-1 illustrates the differences between fertilized and unfertilized eggs.

Sperm transfer and sperm survival

The number of sperm transferred and their survival inside females were measured from the first set of crosses as follows. One female was removed from the cage and dissected one day after the removal of the male and another female was dissected one week after removal of the male. These time points were used to examine whether sperm storage or survival varies between versus within populations. The female's reproductive tract was removed with forceps, the spermathecae were isolated and a 10 µl drop of live/dead stain (Live/Dead Sperm Viability kit, L-7011 from Molecular

Probes, Eugene, OR) was placed on the spermathecae. A cover slip was then positioned over the spermathecae and tapped gently with blunt forceps. This technique releases sperm from the spermathecae (Fry and Wilkinson 2004). The slide was placed on a Nikon Eclipse E600 microscope fitted with two fluorescence filter cubes (B-2E/C and G-2E/C from Nikon) and examined with each to count the number of live (green) and dead (red) sperm stored in each female. The sum of live and dead sperm is used as the total number of sperm transferred.

Sperm motility and storage

Sperm motility and storage were assessed for two females from each cage in the second set of crosses. Females were dissected three days after the male was removed from the cage. The reproductive tract was excised and moved into a drop of PBS and a cover slip was gently placed over it. Sperm was released from the spermathecae by gently placing pressure on the coverslip with blunt forceps. Sperm were then immediately visualized using differential interference contrast (DIC) microscopy at 400X. Motion of live sperm was recorded for 60 seconds using a digital video camera connected to the microscope. Digital video files were subsequently transferred and analyzed on a Macintosh computer. Sperm motility was scored for 10 randomly selected sperm as the number of oscillations per 10 second period using iMovie 3 software. Videos were slowed down to 1/32 speed in order to facilitate counting.

After recording sperm motility, the ventral receptacle (VR) was visualized using oil immersion and DIC microscopy at 1000X. The VR is the short term sperm storage organ, and the site of fertilization (Kotrba 1993). It is comprised of

approximately 50-90 small chambers, each of which is capable of holding a single coiled sperm (Kotrba 1993). The number of VR chambers was counted, as was the number of chambers containing sperm. The proportion of chambers containing sperm was used in the statistical analyses.

Statistical Analysis

Distinguishing among gametic and postzygotic isolation

To determine whether eggs that do not hatch are fertilized or unfertilized, and therefore to distinguish between gametic and postzygotic reproductive isolation (Coyne and Orr 2004), I performed a mixed model ANOVA on the proportion of eggs fertilized or hatched. Because egg fertilization and hatch success could not be measured in the same cross, I combined data from two sets of experimental crosses to perform this analysis. Crosses in which no eggs were fertilized or hatched were excluded from the analysis. Male source population, female source population and their interaction were included in the model as random effects. The type of egg measurement, either "hatched/unhatched" or "fertilized/unfertilized", was included as a fixed effect. The arcsin square-root transformed proportion of eggs fertilized or hatched was the dependent variable. After evaluating the model I used Tukey's honestly significant difference (HSD) post-hoc test to determine if the proportion of eggs fertilized differed from the proportion of eggs hatched within each population cross. If fertilization rate is significantly higher than hatch rate, then some level of hybrid inviability must exist (the alternative test is biologically irrelevant as unfertilized eggs cannot hatch). If there is no significant difference between the

proportion of fertilized and hatched eggs, then there is no evidence of hybrid inviability and, therefore, gametic isolation is implicated as the source of any decrease in fertilization and egg hatch for between population crosses compared to within population crosses.

Reproductive isolating mechanisms

For each mechanism of reproductive isolation, I performed a two-way ANCOVA in order to assess whether the mechanism is affected by male source population, female source population or the interaction of male and female source populations. A significant male by female population interaction effect would indicate that the reproductive isolating mechanism is dependent upon the particular combination of populations being crossed. An effect of just the male or female population would indicate that the reproductive isolating mechanism is controlled by changes in only that sex. The ages of both the male and female were used as covariates. Data for sperm motility and sperm survival were not available for combinations of male and female populations where mating did not occur. In most cases, these crosses involved the Cameron population of *T. dalmanni*. Therefore, the Cameron population was not included in the ANCOVAs for these sperm variables.

I then performed two sets of stepwise multiple regression analyses to examine the relative importance of each reproductive isolating mechanism. To compare mechanisms I used mean values for each cross type so that data from both series of crosses could be used. The dependent variable in these analyses is total eggs hatched per day, which I used to quantify successful reproduction. In the first analysis, I

included all crosses – mated and unmated – and used four independent variables, in the following order: proportion of females with sperm in their reproductive tracts, proportion of VR chambers containing sperm, proportion of eggs fertilized, and proportion of fertilized eggs hatched. The first independent variable, proportion of females with sperm in the reproductive tract, is used as a proxy for mating and therefore represents the relative importance of premating reproductive isolation. The second and third independent variables, proportion of VR chambers containing sperm and proportion of eggs fertilized, represent gametic reproductive isolation. The final independent variable, proportion of fertilized eggs hatched, represents postzygotic reproductive isolation due to hybrid inviability.

A significant proportion of females in heteropopulation crosses, particularly in those crosses involving the Cameron population, did not mate. Therefore I carried out a second stepwise multiple regression excluding crosses in which mating was unsuccessful. I also excluded the first independent variable, proportion of females with sperm in their reproductive tracts, because removing unmated females eliminates variation in this variable. The stepwise analyses described above were then repeated with only three independent variables.

Gametic isolation

To assess the causal relationships among the gametic isolation mechanisms, I performed a multiple regression using log sperm number, sperm viability, sperm motility and proportion of sperm in the VR as independent variables and proportion of eggs fertilized as the dependent variable. This analysis is intended to reveal the step in

the process between mating and fertilization at which incompatibilities exist between populations. Therefore, crosses in which no sperm were transferred were removed. Cross means were used to allow inclusion of multiple datasets, as described above. The cross between *T. dalmanni* Cameron males and *T. dalmanni* Soraya females produced no successful fertilizations and was therefore excluded from the analysis.

Results

Distinguishing between gametic and postzygotic isolation

The overall effect of egg outcome was marginally significant, with mean proportion of eggs fertilized being slightly higher than mean proportion of eggs hatched (DF = 1; F = 4.6; P = 0.03). ANOVA post-hoc tests revealed that hatching and fertilization success were significantly different for two of the 12 between-population crosses – Soraya male mated to Gombak female and Soraya male mated to Bukit Lawang female (Tukey's HSD; alpha = 0.05; power = 0.71). In all remaining crosses, hatch and fertilization success did not differ (Figure 3-2).

Reproductive isolating mechanisms

The results of the two-way ANCOVAs for male and female population on each of the eight variables corresponding to alternative mechanisms of prezygotic isolation are shown in Table 3-1. Least squared means with standard errors for each response variable are shown in Figure 3-3. Five of the response variables – mating, log sperm number, proportion of VR chambers with sperm inside, proportion of eggs fertilized and proportion of eggs hatching – were significantly affected by male population,

female population and the interaction of those terms after sequential Bonferroni correction. Sperm motility and the number of eggs laid were affected only by female population. Sperm viability was not significantly influenced by any of the factors in the model. Neither male nor female age showed a significant effect on any of the reproductive isolating mechanisms.

The results of the stepwise multiple regressions are shown in Table 3-2. The first multiple regression, which included all crosses (regardless of whether mating occurred) showed a highly significant (P = 0.0002) effect of mating on the number of eggs hatched. The proportion of eggs fertilized and the proportion of fertilized eggs (P = 0.04) that hatched (P = 0.02) were also significant. The second regression, which included only crosses in which mating occurred, showed a highly significant (P = 0.0004) effect of proportion of sperm in the VR. The proportion of eggs fertilized was no longer significant and the proportion of fertilized eggs that hatched was marginally significant (P = 0.08 and 0.05, respectively).

Gametic isolation

Multiple regression of the gametic reproductive isolating barriers on the proportion of eggs fertilized revealed a highly significant effect of proportion of VR chambers with sperm (overall model, $R^2 = 0.90$; $t = 6.82$, P = 0.0002) but no effect of log sperm number ($t = -1.03$, P = 0.33), sperm viability ($t = -0.94$, P = 0.37) or sperm motility ($t = 1.11$, P = 0.30). The relationship between proportion of VR chambers with sperm and proportion of eggs fertilized is shown in figure 3-4. Partial correlations between log sperm number, sperm viability, sperm motility and proportion of VR chambers

with sperm revealed no significant correlations (all r values were under 0.5). This lack of significant correlations indicates that mechanisms involving the number or quality of sperm transferred cannot fully explain the decreased ability of sperm to reach the VR in between-population crosses.

Discussion

Among these populations of stalk-eyed flies, I have found that reproductive isolating barriers have evolved at the premating, gametic, and postzygotic levels. I find that decreases in heteropopulation progeny production are driven by decreased mating success, decreased sperm transfer, and decreased ability of sperm to reach the site of fertilization. In conjunction with the results of Christianson *et al.* (2005), which showed that premating behavioral isolation and postzygotic isolation in the form of hybrid inviability are also important among these populations, I conclude that various levels of reproductive isolation are evolving concurrently in this incipient species complex.

Gametic vs. postzygotic isolation

A primary goal of these experiments was to discriminate between gametic isolation and postzygotic isolation in heteropopulation crosses where hatching success has been observed to decrease with genetic distance (Christianson et al. 2005). Christianson *et al.* (2005) found evidence of decreased hatching success between populations. This finding confirmed the presence of reproductive isolation, however it was unclear whether the observed decrease in hatching success was a product of gametic isolation

(i.e. unhatched eggs were unfertilized) or postzygotic isolation (i.e. unhatched eggs were zygotes that failed to develop due to hybrid inviability). To distinguish between gametic and postzygotic incompatibilities, I compared fertilization success to hatching success. If fertilization success is significantly higher than hatching success, then eggs laid by females in this cross are being fertilized but are not hatching. This result would indicate that some postzygotic isolation in the form of hybrid inviability is occurring. However, if no difference between fertilization and hatching success is found, then I can conclude that the decrease in hatching success among populations is driven by gametic isolation, not postzygotic isolation.

I found that only two of the 12 heteropopulation crosses examined have significantly higher fertilization success than hatching success (figure 3-2). Among these two population pairs (Soraya male x Gombak female and Soraya male x Bukit Lawang female), hybrid inviability has evolved, presumably as a result of the accumulation of deleterious epistatic Dobzhansky-Muller incompatibilities (Orr and Turelli 2001). The other ten heteropopulation crosses showed no measurable level of postzygotic reproductive isolation in the form of hybrid inviability. Therefore, I conclude that in these heteropopulation crosses, when eggs fail to hatch, it is due to a lack of successful sperm-egg fusion rather than hybrid inviability. This result validates further investigation into the mechanisms of gametic isolation among these populations.

Mechanisms of non-competitive gametic isolation

I measured eight potential variables related to non-competitive gametic isolation: mating (categorical), number of sperm transferred, sperm motility, sperm survival, sperm storage success, number of eggs laid, proportion of eggs fertilized and proportion of eggs hatched. Each of these mechanisms was found to increase reproductive incompatibility as population divergence increases (figure 3-3). Five of these reproductive isolating mechanisms – mating, sperm number, proportion of VR chambers with sperm inside, proportion of eggs fertilized and proportion of eggs hatching – were significantly affected by the interaction of male by female population of origin effect. These mechanisms depend, therefore, on the particular combination of male and female (or ejaculate and female reproductive tract) and are not determined by one sex alone. Conversely, sperm motility and the number of eggs laid were affected only by female population. The female-only effect on sperm motility is surprising, and intimates possible differences in the chemical composition of the female reproductive tract among these populations of stalk-eyed flies (Eberhard 1996). Sperm viability was not significantly affected by male or female population, their interaction effect, or male or female age.

The Cameron population of *T. dalmanni*, which exhibits over 5% divergence (based on partial mtDNA sequences of cytochrome oxidase II and the large ribosomal subunit) from the other three *T. dalmanni* populations used in this study (Swallow et al. 2005), showed nearly complete reproductive isolation from the other populations. In most crosses between Cameron and the other *T. dalmanni* populations, the primary cause of reproductive isolation was decreased sperm transfer. One notable exception was the Cameron male by Gombak female cross (CG in figure 3-3), in which some

mating occurred, sperm were transferred and those that survived were relatively motile. However, Cameron sperm did not survive for long in the Gombak female reproductive tract; the average proportion of live sperm to dead sperm in the female reproductive tract for this cross was approximately 0.33, compared to a conpopulation survival rate of approximately 0.93. Consequently, the VR was not populated with sperm; only 13% of VR chambers had sperm in them, compared to a within population rate of 91%. In this cross only 1.5% of all eggs laid by the female were fertilized successfully. This particular cross provides an illustration of nearly complete reproductive isolation evolving as a result of multiple partial reproductive incompatibilities.

The best predictor of hatching success was whether or not sperm are present in the female reproductive tract (table 3-2, "all crosses"). If no mating occurs, then no sperm are transferred and no progeny are produced. This finding confirms the result from Christianson *et al.* (2005), which showed a significant level of premating reproductive isolation among these populations. Consequently, in order to examine postmating reproductive isolation, I excluded unmated females from the analyses.

After excluding unmated females, the ability of sperm to reach the site of fertilization, the VR, was found to have a highly significant effect on hatching success (table 3-2, "unmated females excluded"). If sperm from heteropopulation males was not stored in the VR, hatching success was low. Additionally, successful sperm storage in the VR was the best predictor of fertilization success in population crosses with sperm transfer (figure 3-4). The multiple regression analysis also revealed that number of sperm transferred, sperm viability and sperm motility did not have a

significant effect on proportion of eggs fertilized. The decrease in proportion of VR chambers with sperm between populations was not caused by decreased sperm transfer, sperm motility or sperm viability.

The process of fertilization in stalk-eyed flies requires movement of sperm from the internal spermatophore, up the spermathecal ducts, into the spermathecae and then back down the ducts and over to the VR for short-term storage and eventually fertilization (Kotrba 1993). Postcopulatory sexual selection in the form of sperm competition and/or cryptic female choice has played a role in the evolution of these organs and has driven divergent selection among these allopatric populations (cf. Chapter 2). Further evidence of the evolutionary importance of the VR in stalk-eyed flies has been found in previous studies (Kotrba 1993). Among genera of stalk-eyed flies, morphology of the VR is highly diversified and the size of the VR is positively correlated to sperm length across taxa (Presgraves et al. 1999). My finding corroborates the significant role of this female reproductive organ and demonstrates that it has the potential to generate reproductive isolation among closely related populations.

Simultaneous evolution of reproductive isolating barriers

Each heteropopulation cross exhibits a unique combination of reproductive isolating barriers. Among more distantly related populations, premating isolation is the primary barrier observed. This is expected as a lack of mating or mate attraction is the endpoint of speciation as defined by the biological species concept (Coyne and Orr 2004) and inherently prevents "downstream" reproductive isolating barriers from

occurring even if incompatibility at those barriers exists among populations. However, I find that among more closely related populations where speciation is not yet complete, reproductive isolation is evolving simultaneously through multiple modes.

Thus, my results in combination with the findings of Christianson *et al.* (2005) support an emerging trend in the study of reproductive isolation: that multiple reproductive isolating barriers drive speciation among a single pair of populations (Coyne and Orr 1997; Dopman et al. 2010; Malone and Fontenot 2008; Matsubayashi and Katakura 2009; Stelkens et al. 2010). I have not measured ecological speciation in this species complex, as these are laboratory populations. However, every other type of reproductive isolation that has been measured among these populations of *Teleopsis* has been detected to influence gene flow between at least one population pair. Stochastic or selective evolutionary divergence among allopatric populations occurs on many traits simultaneously, and this divergence leads to an array of incompatibilities upon secondary contact.

Tables

Table 3-1. Results from ANCOVAs of reproductive isolating mechanisms. F-values of male and female population and their interaction effects with age are shown. Significance corrected by sequential Bonferroni. *** = P < 0.0001.

Dependent variable	Male Population	Female Population	Male x Female Population	Male Age	Female Age
Mating	18.7***	9.6***	16.0***	0.0	0.1
Log Sperm Number	7.3***	8.7***	19.3***	0.3	1.1
Sperm Survival†	0.7	0.8	0.6	1.0	3.0
Sperm Motility†	1.0	6.6*	1.1	0.8	1.3
Proportion VR Full	35.3***	32.7***	48.7***	1.2	1.7
Number of eggs laid	0.2	54.4***	1.4	0.0	0.9
Proportion Eggs Fertilized	27.4***	26.8***	60.2***	0.7	0.7
Proportion Eggs Hatched	23.7***	12.7***	44.9***	4.1	1.9

† excludes crosses involving the Cameron population

Table 3-2. Results of sequential multiple regressions of reproductive isolating mechanisms on total eggs hatched per day.

Variables in Model	All crosses		Unmated females excluded	
	F	P value	F	P value
Proportion mated	29.1	0.0002	-	-
Prop. of sperm in VR	0.8	0.38	26.3	0.0004
Prop. of eggs fertilized	5.7	0.04	3.7	0.08
Prop. of fertilized eggs that hatched	6.8	0.02	5.2	0.05

Table 3-3. Partial correlations between mechanisms of gametic isolation on fertilization success. Average proportion of VR chambers with sperm is highly correlated to proportion of eggs fertilized.

	Proportion of eggs fertilized	Sperm Motility	Average proportion of VR chambers with sperm
Proportion of eggs fertilized	1		
Sperm Motility	0.1326	1	
Average proportion of VR chambers with sperm	0.7347	0.2078	1

Figures

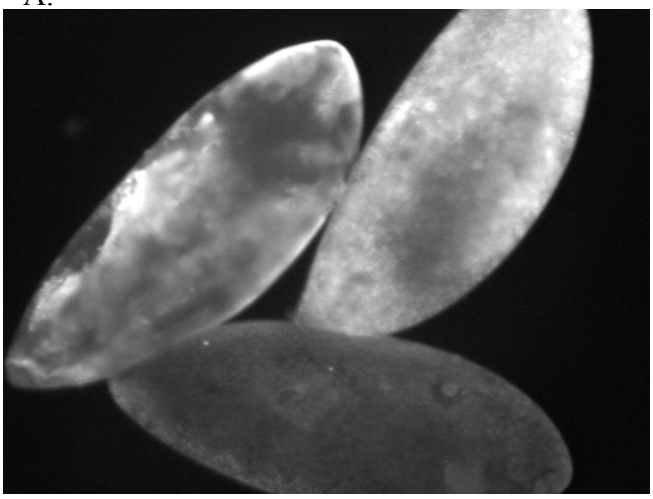
Figure 3-1. Images of unfertilized (A) and fertilized (B and C) eggs. Eggs were stained with 10^{-7} Hoechst 33258 and photographed under UV fluorescence. The fertilized eggs show evidence of cellular development.

Figure 3-2. A comparison of proportion of eggs fertilized (dark gray with hatch marks) and proportion of eggs hatched (light gray) by population cross. Least squares means +/- S.E. are shown. Population cross designations are listed as "male population, female population". C = Cameron, G = Gombak, L = Bukit Lawang, S = Soraya. If proportion of eggs fertilized is higher than proportion of eggs hatched, then postzygotic incompatibilities are occurring (i.e. fertilized eggs are not developing successfully). In contrast, if the proportion of eggs fertilized is similar to the proportion of eggs hatched, postzygotic incompatibilities are not likely to be influential. Proportion of eggs fertilized was significantly higher than proportion of eggs hatched for two populations crosses, S,G and S,L – these crosses are designated with an asterisk.

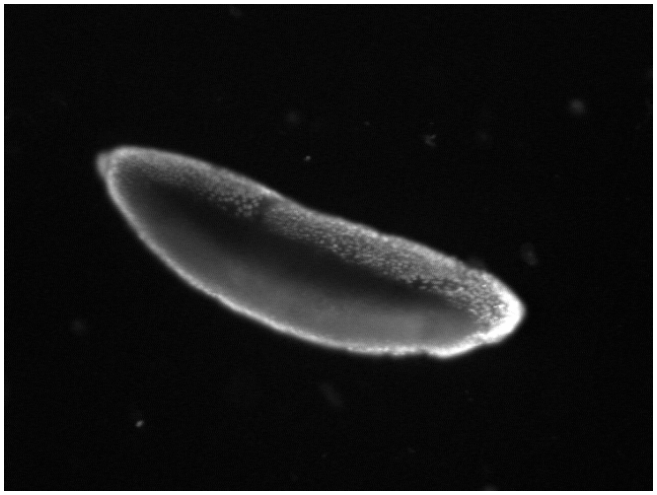
FIGURE 3-3. Reproductive isolating mechanism means by cross name. Cross names, on the x-axis, are organized by increasing genetic distance. The first four crosses are within population. Male population is listed first, for example, "LS" is a cross between a Lawang male and a Soraya female.

Figure 3-4. Results of the regression of proportion of fertilized eggs on proportion of sperm in the VR (overall model: $R^2 = 0.90$; $t = 6.82$, $P = 0.0002$). This significant association indicates a relationship between the inability of heteropopulation sperm to reach the VR, which is the site of fertilization, and the decrease in fertilization success across populations.

A.



B.



C.

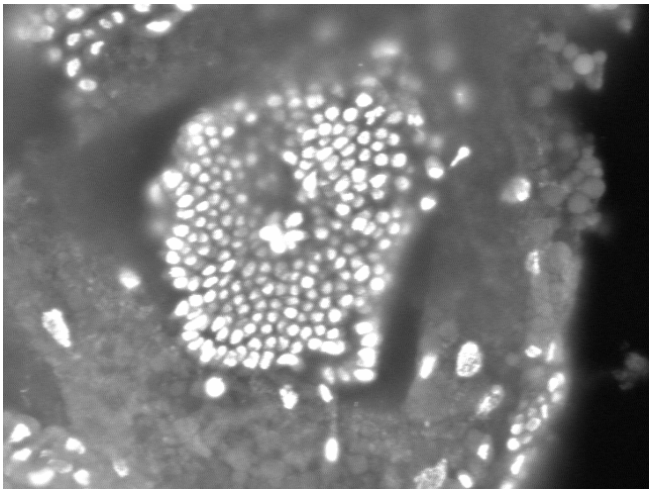


Figure 3-1

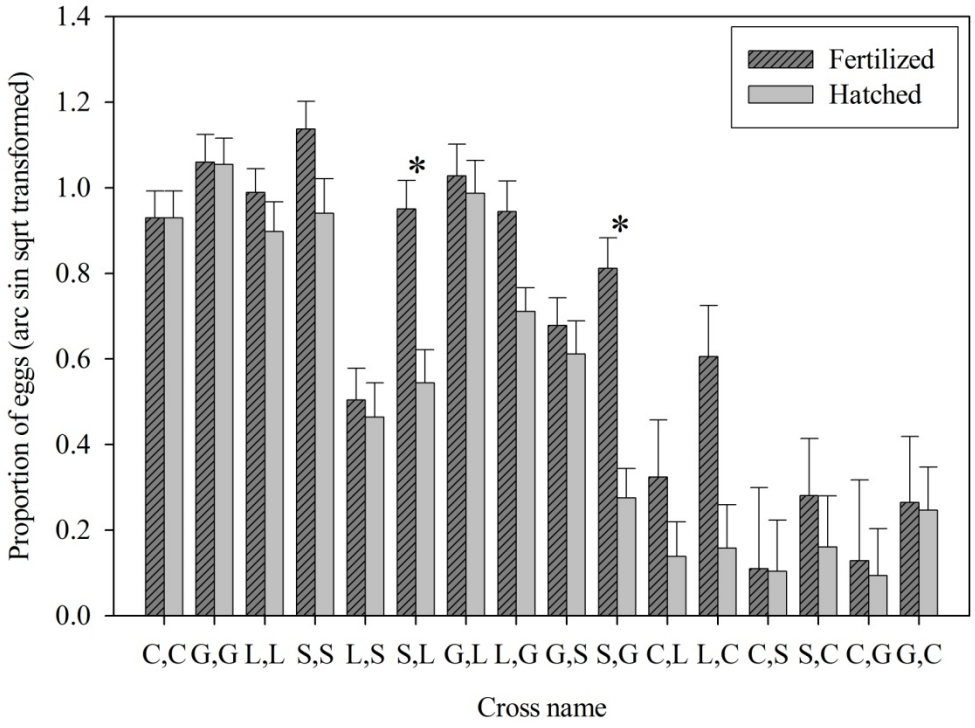

Fertilized
Hatched
Proportion of eggs (arc sin sqrt transformed)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
C,C G,G L,L S,S L,S S,L G,L L,G G,S S,G C,L L,C C,S S,C C,G G,C
Cross name

Figure 3-2

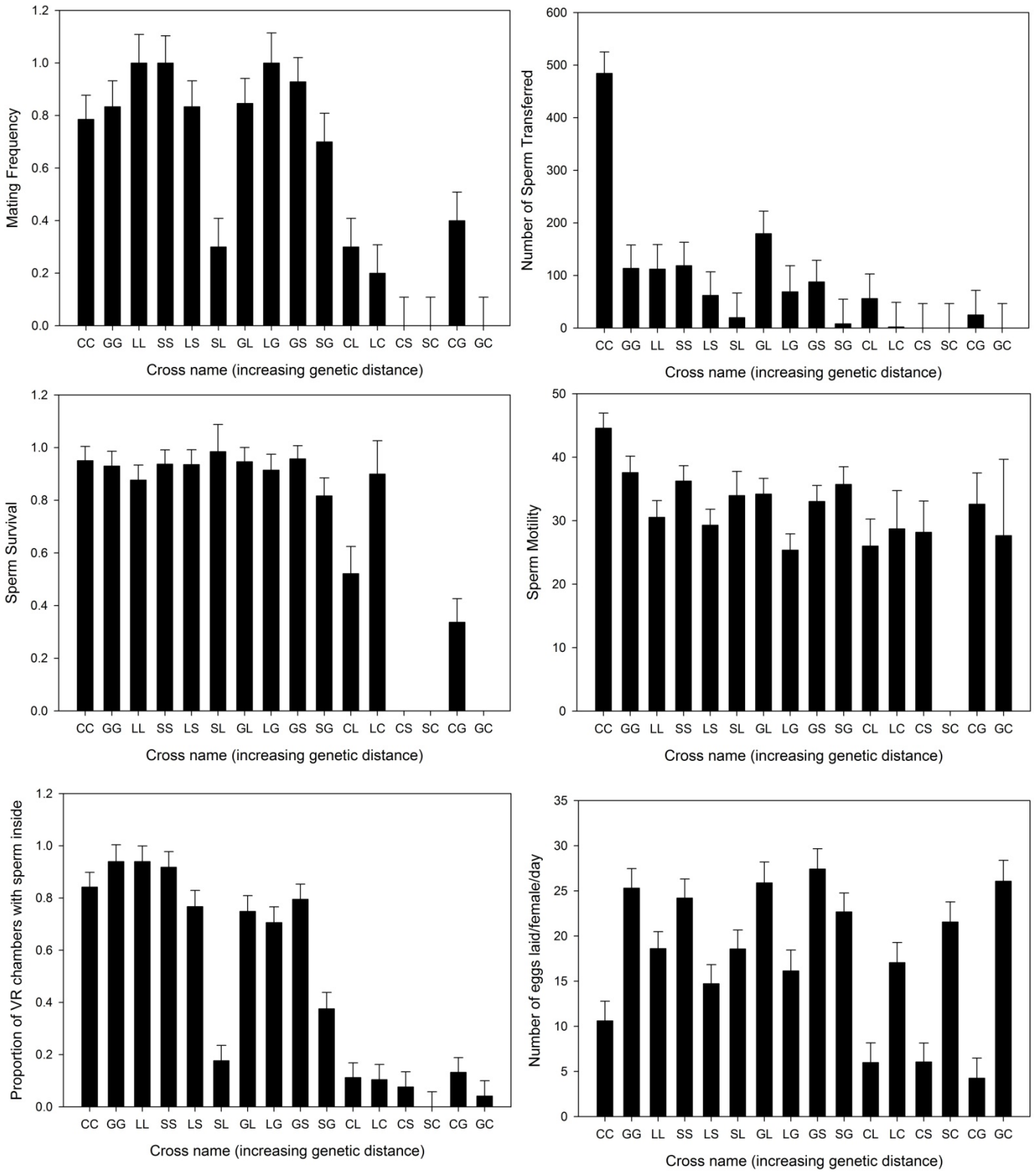

Mating Frequency
Number of Sperm Transferred
Sperm Survival
Sperm Motility
Proportion of VR chambers with sperm inside
Number of eggs laid/female/day
CC GG LL SS LS SL GL LG GS SG CL LC CS SC CG GC
Cross name (increasing genetic distance)

Figure 3-3

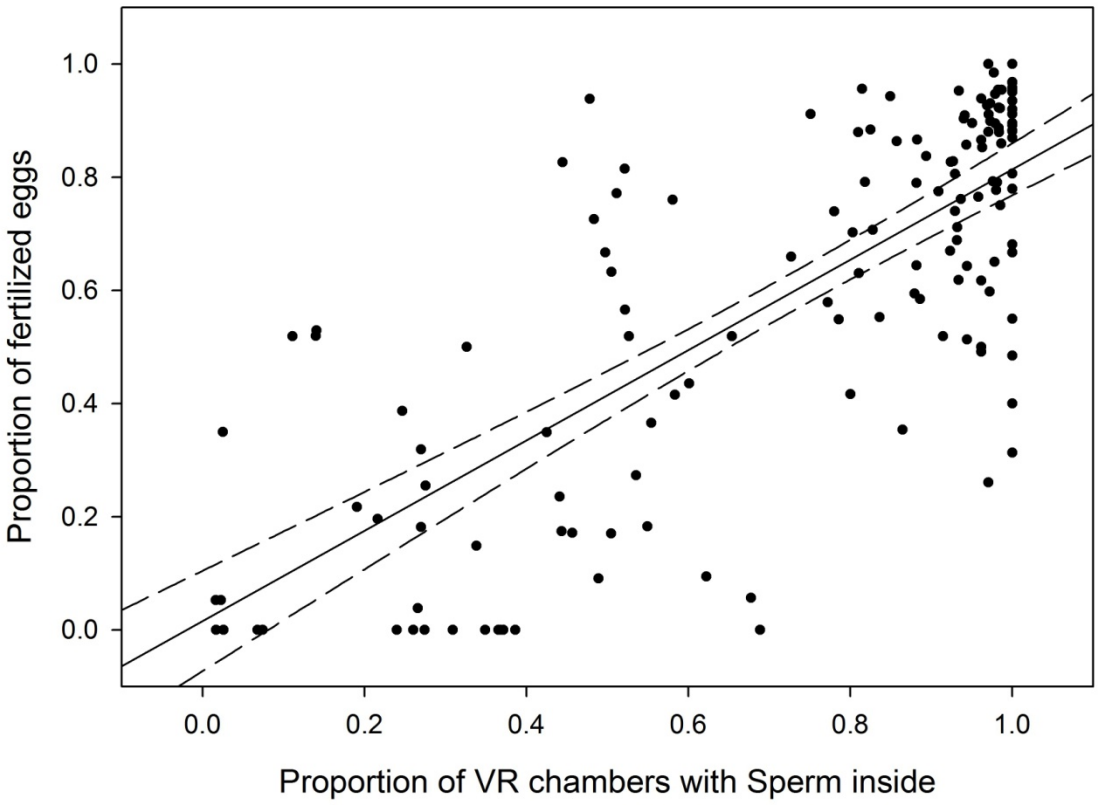

Proportion of fertilized eggs
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.2
0.4
0.6
0.8
1.0
Proportion of VR chambers with Sperm inside

Figure 3-4

Chapter 4: Competitive Gametic Isolation in Stalk-Eyed Flies

Abstract

The influence and importance of postcopulatory sexual selection as a driving force of speciation is an area of increasing interest to evolutionary biologists. Gametic isolation is the form of reproductive isolation that involves barriers to gene flow after mating and before fertilization. Here I examine competitive gametic isolation – which occurs when sperm from different populations compete for fertilization – among two pairs of isolated populations of stalk-eyed flies. I find evidence of conpopulation sperm precedence between both pairs of populations – and my conclusions are enriched by the inclusion of sperm transfer data from single population crosses. Of the eight crosses in which heteropopulation males were competing for fertilizations, four provided clear evidence for conpopulation sperm precedence, one exhibited a pattern of heteropopulation sperm precedence, and three crosses showed no deviation from the expectation of complete sperm mixing. I conclude that competitive gametic isolation has the potential to contribute to reproductive isolation between these populations. I suggest further study on the relative importance of gametic isolation in comparison to other reproductive isolating barriers in prohibiting gene exchange and driving speciation among these populations.

Introduction

In recent decades, studies of sexual selection have evolved to include interactions between the sexes that occur after mating (Parker 1970). Postcopulatory

sexual selection has been established as an important evolutionary force capable of causing rapid evolution of female reproductive tracts (Eberhard 1996), sperm (Simmons 2001), and seminal products (Coyne and Orr 2004; Eady 2001; Howard 1999). More recently, interest in the power of these postcopulatory interactions to create barriers to gene flow among populations has arisen. Dobzhansky (1937) first recognized that incompatibility at the gametic level could contribute to reproductive isolation. However, this possibility has received little investigation until recently (Coyne and Orr 2004).

Gametic isolation is a mechanism of reproductive isolation that occurs at any stage between mating and formation of the zygote. Coyne and Orr (2004) divide gametic isolating mechanisms into two categories: non-competitive and competitive. Non-competitive gametic isolation involves the inability of heterospecific or heteropopulation sperm to achieve fertilization in the absence of sperm competition. Some mechanisms which cause non-competitive gametic isolation include poor transfer or storage of sperm, inviability or decreased motility of sperm in the female reproductive tract, inability of sperm and egg to fuse, or failure to stimulate oviposition (Coyne and Orr 2004). The most convincing evidence of reproductive barriers evolving by non-competitive gametic isolation is from sperm-egg incompatibilities in externally spawning organisms. Studies of the evolution of sperm and egg recognition molecules in abalones have found that these molecules are highly species specific and evolve rapidly by positive selection (Kresge et al. 2001). Broadcast spawning is a simplified fertilization system. In species with internal fertilization males face the added challenge of transferring sperm successfully, which

can involve the gametes navigating convoluted ducts of the female reproductive tract (Eberhard 1996) and stimulating oviposition. Price *et al.* (2001) found that single copulations among three species of the *Drosophila simulans* complex revealed three separate types of noncompetitive gametic isolation acting among them, all involving sperm transfer and storage inefficiencies.

I explored the importance of non-competitive gametic isolation among populations of the stalk-eyed fly, *Teleopsis dalmanni*, in chapter 3. My findings indicate that non-competitive gametic isolation is an important barrier to reproduction among populations. In particular, I identified the inability of heteropopulation sperm to reach the site of fertilization as a mechanism of non-competitive gametic isolation in stalk-eyed flies. Non-competitive and competitive gametic isolation need not be mutually exclusive and have been found to occur simultaneously in several systems (Brown and Eady 2001; Price 1997; Price et al. 2001).

Here I test for competitive gametic isolation among populations of stalk-eyed flies. Competitive gametic isolation occurs when a female mates with both a conspecific and a heterospecific male and these ejaculates overlap in time and space. In this scenario, sperm from each male type is physiologically capable of fertilizing the ova (Gregory and Howard 1994), but this competitive situation results in one male type achieving more fertilizations than the other as a result of sperm competition. This pattern has been well documented in nature and is commonly referred to as conspecific sperm precedence (Chang 2004; Dixon et al. 2003; Geyer and Palumbi 2005; Gregory and Howard 1994; Howard 1999; Price 1997; Rieseberg et al. 1995; Wade et al. 1994). There are several evolutionary explanations that can be used to

predict the outcome of such a cross. Cryptic sexual selection predicts that the conspecific male is best adapted to his mate and thus will sire the majority of her offspring. This outcome may be due to sperm competitive advantages of the conspecific male, cryptic female preference for the conpopulation male, or a combination of the two (Howard 1999). In contrast, sexual conflict theory predicts that males from closely related but different populations or species will have an advantage in sperm competition because females will not have evolved to resist their manipulations (Andres and Arnqvist 2001; Hosken et al. 2002; Rice 1998). This advantage will come at some fitness cost to females. Empirical findings more commonly support conspecific sperm precedence over heterospecific sperm precedence. However, some examples of heterospecific sperm precedence have been found (Hosken et al. 2002; Tregenza and Wedell 2002).

While multiple studies have shown that competitive gametic isolation decreases gene flow in plants (Campbell et al. 2003), marine invertebrates (Geyer and Palumbi 2005), and insects (Fricke and Arnqvist 2004), few of them have accounted for the effects of non-competitive gametic isolation when examining the importance of competitive gametic isolation as a barrier to gene flow. This is problematic because conspecific sperm precedence may be confounded by non-competitive isolation. In such cases, differential offspring production may be due, for example, to decreased sperm transfer from heterospecific males rather than a fertilization advantage for conspecific males. Therefore, a result of "conspecific sperm precedence" in which heteropopulation males produce only 25% of the offspring may be caused entirely by a 50% decrease in sperm transfer between the species. Conversely, if more sperm are

transferred in heteropopulation than conpopulation crosses, instances of conspecific sperm precedence may be masked by ignoring non-competitive factors.

In this study, I carry out a sperm competition experiment in which three allopatric populations of *T. dalmanni* are interbred to determine whether heteropopulation ejaculates are at a competitive disadvantage when competing for fertilizations against conpopulation males. I analyze the data both before and after controlling for the number of sperm transferred in single heteropopulation crosses (as measured in chapter 3). I am thus able to evaluate the presence of conpopulation sperm precedence among these populations and avoid conflating non-competitive (i.e. sperm precedence due solely to relative sperm number) and competitive gametic isolation. In accordance with convention, I describe paternity patterns in terms the proportion of offspring sired by the second male, P2.

The stalk-eyed fly populations that are crossed in this study were chosen based on their demonstrated ability to produce offspring in single heteropopulation matings (chapter 3). These population pairs show little or no reduction in the number of offspring produced in single heteropopulation matings, so that a competitive postcopulatory environment is probable. Using data from my study of non-competitive gametic isolation, I test for a correlation between the observed data and an *a priori* prediction of the percentage of offspring sired by each male assuming complete sperm mixing. If such a correlation is observed, I can conclude that non-competitive gametic isolation accounts for some of the observed variation in paternity. The comparison between observed and expected paternity of each male type can then

inform my conclusion about the presence of competitive gametic isolation among these populations.

Methods

Fly collection and maintenance

I used laboratory stocks of three allopatric populations of *Teleopsis dalmanni* collected from diverse geographic locations on the Sunda Shelf region of Southeast Asia (Swallow et al. 2005). Over a five-year period, from 1996 – 2000, stalk-eyed flies were collected by hand net near streams from nine sites in Thailand, peninsular Malaysia, and the islands of Java, Sumatra, and Borneo. In August 1999 a population of *T. dalmanni* was captured at Ulu Gombak, Malaysia (3°12′N, 101°42′E) and a different population was collected from the Soraya field station on Sumatra (2°52′N, 97°54′E). In September 2000 another population of *T. dalmanni* was collected near Bukit Lawang, Sumatra (3°35′N, 98°6′E). These populations are now referred to as *T. dalmanni* Gombak, Soraya and Bukit Lawang. Since the time of collection, laboratory populations have been maintained in the lab in large cages at 25^{o}C and 70% relative humidity on a 12L:12D. The experiments described here were conducted in 2007 and 2008.

I performed all possible double-mated crosses between two pairs of populations – Gombak/Soraya and Gombak/Bukit Lawang. This design enables me to tease apart the effects of male order from the effects of maternal and paternal population of origin. Individuals of all populations were collected from breeding cups placed in the population cages and reared under standard conditions. Males and

females were separated prior to sexual maturity at three weeks of age (Baker et al. 2003) so that experimental females were virgins upon mating with the first experimental male. To ensure that no experimental females carried any stored sperm, a sample female from each cage was dissected and her reproductive tract was examined for the presence of sperm. If sperm were found, the entire cage of females was discarded. If no sperm were present, females from that cage were assumed to be virgins and subsequently used in experimental crosses.

Mating protocol

There are eight possible cross types between a pair of populations when two males are mated to one female. I carried out every combination of first male, second male and female. Throughout this chapter, cross types will be denoted as [Male 1 population][Male 2 population]_[female population]. For example, if a female from population A was mated to a male from population A and then a male from population B, the cross type is denoted: AB_A. The eight possible cross types are: AA_A, AB_A, BA_A, BB_A, AA_B, AB_B, BA_B, and BB_B. Population names have been abbreviated as follows: Gombak (G), Soraya (S) and Bukit Lawang (L).

Each of the eight cross types was replicated 10 times for both population pairs, giving a total of 160 experimental replicates. For each replicate, one virgin female and one male, both of known age, were placed in a cage and left to copulate freely for three days. The male was removed after the three day period and frozen for later genotyping. A second male was subsequently placed in the cage with the female, along with a fresh cup of food, and they were also allowed to mate freely for a three

day period. The second male was also removed and frozen for later genotyping. The experimental female was allowed to lay eggs for a period of one week following removal of the second male.

Offspring collection and genotyping

Food cups containing 50 ml of pureed corn were provided to females for oviposition and were changed once, mid-week. They were then placed in an incubator set for 12:12 LD at 25^{o} C. Pupae were extracted from these cups as they emerged and were placed in a 500 ml cup lined with moist cotton in the same incubator. These cups were checked daily for offspring eclosion. All offspring were collected daily and frozen for later genotyping. To minimize the amount of unnecessary genotyping, I first sampled ten offspring per female, approximately one-third of the total number of eggs laid – females produce 2-3 eggs per day, on average (Wilkinson et al. 2006; Wright et al. 2004). If all 10 of the sampled offspring were sired by one male, I inferred that this male sired all of the offspring. If a mixture of both paternal genotypes was detected, indicating mixed paternity, then another sample of ten offspring was genotyped (if enough offspring existed) and the proportion of offspring sired by each male was determined based on this sample of 20 offspring.

Potential parents and offspring were genotyped at three highly informative microsatellite loci (Wright et al 2004). If paternity could not be successfully assigned based on those loci, then up to six additional loci were typed, until parentage could be inferred. DNA was extracted from the mother, both putative fathers and offspring using a Qiagen DNeasy extraction kit. DNA was amplified and genotyped via PCR

using autosomal microsatellite loci: 174, 249, and 402a (Wilkinson et al. 2006). If paternity could not be determined from those initial loci, the following six microsatellite loci were amplified: 402b, 301, 301a, 90, 262z, and 39p. PCR products were separated on an ABI 3730xl DNA analyzer and Genemapper v.4 was used to score fragment length. A total of 1257 progeny from 117 females were collected and genotyped. Paternity was assigned by the presence of at least one unique PCR product shared between a male parent and offspring. In total, paternity was successfully assigned to each male in 92.9 ± 1.3% of broods.

Statistical Analyses

Analysis of second male sperm precedence (P2)

To determine whether heteropopulation males were less successful at producing offspring when in competition with conpopulation males, I carried out a 2-way ANOVA using "cross type" categories (AA_A, AA_B, AB_A, BA_A) and "population pair" (G-L or G-S) as factors. The interaction between cross type and population pair was included in the model. Conpopulation sperm precedence predicts that P2 for the AA_A and AA_B cross types – those in which males from the same population are in competition – will be intermediate, while AB_A crosses are expected to have low P2 and the BA_A crosses are expected to have high P2. Once a significant effect of the interaction between cross type and population pair was found, a Tukey's HSD test was conducted to identify statistical differences among the cross type levels.

Test for effect of non-competitive gametic isolation on conpopulation sperm precedence

In order to account for the effects of non-competitive gametic isolation, I compared expected to observed P2 for each cross. To establish expected P2, I utilized the data from chapter 3 on the number of sperm transferred in non-competitive single crosses between populations. I then calculated the proportion of sperm expected to be transferred to a female of each population after mating with a male from both populations and assuming that complete sperm mixing alone had occurred. This assumption is supported by several prior studies of sperm precedence in stalk-eyed flies demonstrating a pattern of sperm mixing (Lorch et al. 1993; Wilkinson et al. 2006). For example, for the SG_G cross in which a female from the Gombak population was mated first to a Soraya male and then to a Gombak male, I divided the average number of sperm transferred in a single Gombak male/Gombak female cross (113) by the sum of the sperm transferred in a single Soraya male/Gombak female cross (88) and in a single Gombak male/Gombak female cross (113) to get P2, the expected proportion of offspring sired by the second male, (in this case, 113/[88+113] = 0.56). This value was used as expected P2. For crosses in which the males were from the same population, expected P2 was set to 0.5.

I then carried out a linear regression of observed P2 on expected P2. The amount of variation in observed P2 explained by expected P2 indicates the extent to which non-competitive gametic isolation can account for apparent conspecific sperm precedence. A high R^2 value, therefore, would suggest that much of the variation in P2 observed in the present study is due to variation in the number of sperm transferred

and should not be attributed to sperm competition. Conversely, a low R^2 value would reveal that non-competitive gametic isolation did not contribute to differences in P2 among populations.

Effect of female population

To determine whether female population of origin affects sperm precedence I conducted an ANOVA using only the crosses in which both males were from the same population. Crosses were categorized as either conpopulation (i.e. AA_A) or heteropopulation (i.e. AA_B) and this categorical variable was included to remove the confounding effect of crosses in which males are from a different population than the female. I tested for an interaction between female population and cross type. A significant effect would indicate that female reproductive tract divergence among populations has altered the environment in which sperm compete.

Results

Detection of conpopulation sperm precedence

The 2-way ANOVA revealed a significant main effect of cross type ($F = 16.2$, $P < 0.0001$, $DF = 3$) and a significant interaction between cross type and population pair on P2 ($F = 9.0$, $P < 0.0001$, $DF = 3$). Results of Tukey's HSD post-hoc tests (Figure 4-1) conform to predictions of conspecific sperm precedence. The crosses in which both males are from the same population (AA_A and AA_B) have intermediate P2 values. The AB_B crosses, in which the first male is heteropopulation and the second male is conpopulation, have significantly higher mean P2. Finally, the AB_A crosses, with a

conpopulation first male and heteropopulation second male, show very low P2. Therefore, it appears that conpopulation males have a sperm competitive advantage over heteropopulation males regardless of male order. The significant interaction of population pair and cross type indicates that the GS crosses and the GL crosses have different patterns of P2 (Figure 4-2a and 4-2b).

Effect of non-competitive gametic isolation on conpopulation sperm precedence

The regression analysis shows a significant effect of expected on observed P2 ($P = 0.02$, $R^2 = 0.37$). Expected P2, which was calculated using the number of sperm transferred in single population crosses and sperm mixing as a proxy for non-competitive gametic isolation, therefore explains 37% of the variance in observed P2 across all crosses.

Effect of female population on conpopulation sperm precedence

I did not find a significant effect of female population ($F = 0.66$, $P = 0.48$), cross type ($F = 0.00$, $P = 0.98$), or the interaction between female population and cross type ($F = 1.79$, $P = 0.18$) on P2.

Discussion

I have found evidence supporting conspecific sperm precedence between both pairs of *Teleopsis* stalk-eyed fly populations examined here. The ANOVA of cross type and population pair on P2 indicates that sperm competition generally favors the conpopulation male as predicted by conpopulation sperm precedence (Figure 4-1).

However, I also found that a significant amount of variation in P2 can be attributed to non-competitive gametic isolation. Specifically, 37% of variation in observed P2 was explained by number of sperm transferred in single population crosses, under the assumption that sperm mixing occurs (Lorch et al. 1993; Wilkinson et al. 2006). This result illustrates that studies of competitive gametic isolation that neglect to control for the impact of non-competitive gametic isolation may lead to inaccurate conclusions.

When I take into account the expected number of sperm transferred and its potential to affect P2, my conclusions are altered. Examination of expected and observed P2 (Figure 4-2) reveals that some of the apparent conpopulation sperm precedence involving the Gombak and Soraya population crosses can be explained by sperm mixing. Below is a break-down of the pattern of P2 for each cross and an interpretation of the observed patterns as they relate to gametic isolation.

There are five crosses in which expected and observed P2 are similar – GG_L, GL_G, GG_G, GS_S, and SG_S. For these crosses, I conclude that sperm mixing alone is sufficient to predict the pattern of second male sperm precedence. Therefore, no conpopulation or heteropopulation sperm precedence is occurring. These crosses demonstrate the importance of accounting for non-competitive gametic isolation in measuring competitive gametic isolation. The SG_S cross provides an extreme example of why the inclusion of sperm number from non-competitive crosses has helped inform my evaluation of conpopulation sperm precedence. As shown in Figure 4-2, observed P2 (dark gray bar) for this cross was very low (0.07). Had I not accounted for sperm transfer, I would have concluded that the heteropopulation second male was at a severe disadvantage in sperm competition. However, expected

P2 based on sperm transfer data (light gray bar) for this cross is 0.04. Therefore, the observed data do not indicate conspecific sperm precedence. In fact, there is likely little chance of sperm competition occurring among these males because the likelihood of sperm transfer is quite low.

There are nine crosses in which observed P2 was greater than expected P2: GL_L, LG_G, LG_L, LL_G, LL_L, GG_S, SG_G, SS_G, SS_S. Three of these crosses, GL_L, LG_G and SG_G are examples of conspecific sperm precedence. These crosses show higher P2 than expected by sperm mixing alone and represent examples of a second, conpopulation male competing against a heteropopulation male (cross type category AB_B). Therefore, I can conclude that the male from the same population as the female has a sperm competitive advantage over the heteropopulation male. These are clear cases of conspecific sperm precedence, in which sperm competition would increase reproductive barriers between populations in the wild upon secondary contact.

Interestingly, one cross in which observed P2 is greater than expected P2, LG_L, shows a pattern of heteropopulation sperm precedence. In this cross, the heteropopulation second male was more successful at producing progeny than predicted by sperm mixing. This unexpected result may indicate that sexual conflict is occurring within populations. Sexual conflict theory predicts heteropopulation sperm precedence as a result of an antagonistic intrapopulation arms race in which male ejaculates evolve to manipulate females and female reproductive tracts and postcopulatory behavior evolve to resist these manipulations (Arnqvist et al. 2000; Tregenza et al. 2000). The prediction with regards to reproductive isolation is that

females from closely related populations will be naïve with regards to recent male manipulations and these males will be more successful at achieving fertilizations than conpopulation males. However, evidence for this phenomenon is limited (Coyne and Orr 2004) and given the large number of crosses performed here, a more parsimonius interpretation is that the P2 estimate for the LG_L cross represents a chance event. I suggest further study of this population pair before conclusions regarding sexual conflict and reproductive isolation are made.

The remaining five crosses, for which observed P2 was higher than expected P2 – LL_G, LL_L, GG_S, SS_G, and SS_S – are cases in which both of the males were from the same population. Expected P2 was calculated on the assumption of sperm mixing based on previous studies of stalk-eyed flies (Lorch et al. 1993; Wilkinson et al. 2006). It appears that for these five crosses, either the expectation of sperm mixing was not met or measurement/experimental error caused a deviation from that expectation. However, the fact that observed P2 was higher than expected P2 in all of these crosses suggests that the second male may have a small inherent sperm competitive advantage in stalk-eyed flies. Based on this observation, I re-analyzed the data using expected P2 calculated with an expectation of 0.65 (the average within-population P2 observed here) instead of 0.5 (which represents sperm mixing). This change in how I calculated expected P2 had no affect on any of the results or conclusions – the r^2 value of observed P2 on expected P2 for that analysis was 0.38.

There is one cross in which observed P2 was lower than expected P2. This cross, GS_G, provides another clear example of conspecific sperm precedence. Expected P2 based on sperm number and the expectation of sperm mixing was 0.44

and observed P2 was 0.03. Therefore, the heteropopulation second male failed to produce as many offspring as predicted suggesting that the conpopulation first male's sperm out-competed the sperm of the second male. This sperm competitive advantage would potentially drive reproductive isolation upon secondary contact in nature.

Conpopulation sperm precedence is predicted to evolve as a by-product of intrapopulation postcopulatory sexual selection (Coyne and Orr 2004). Based on my finding that female population did not explain a significant amount of variance in P2, I cannot conclude that divergence in female reproductive tract morphology or female postcopulatory behavior has impacted the sperm competitive environment. Therefore, divergence among populations in the content of the male ejaculate is likely responsible for the observed cases of conpopulation (and the one case of heteropopulation) sperm precedence. Many studies have demonstrated that divergence in ejaculate characteristics, such as accessory gland proteins and sperm length, contributes to reproductive isolation among closely related populations across taxa (Aagaard et al. 2010; Birkhead and Brillard 2007; Moy et al. 2008; Panhuis et al. 2003; Pitnick et al. 2003b; Ramm et al. 2009).

My results, in combination with the results of the previous chapter, indicate that both non-competitive and competitive gametic isolation have the potential to decrease gene exchange between these populations of stalk-eyed flies. In conjunction with Christianson *et al.* (2005), there is now evidence that nearly all potential mechanisms for reproductive isolation exist to some extent among these *Teleopsis* populations. This conclusion is consistent with the recent direction of speciation research, in which findings are commonly implicating multiple "leaky" barriers to

reproductive isolation which, in combination, evolve into complete barriers to gene flow (Jennings et al. 2011; Matsubayashi and Katakura 2009; Sobel et al. 2010). If concurrent evolution of numerous incomplete reproductive isolating barriers is commonplace, then conclusions about the importance of a given mode of reproductive isolation should not be made until all levels of reproductive isolation have been studied.

A new interest in defining the relative importance of each mode of reproductive isolation has emerged (Coyne and Orr 2004; Sobel et al. 2010). As described above, available evidence indicates that every category of reproductive isolation exists to some degree among populations of *Teleopsis*, including premating, gametic, and postzygotic (Christianson et al. 2005). An examination of the relative contribution of each barrier – from mating to successful offspring production – to reproductive isolation, would be a compelling direction for future research.

Figures

Figure 4-1 – Results of the Tukey HSD test from the 2-way ANOVA of cross type and population pair on P2. Mean (LSM) and Standard Error (S.E.) of second male paternity (P2) for each cross type is shown along with Tukey's HSD significance levels. The results conform to predictions of conspecific sperm precedence. The AA_A and AA_B crosses, in which both males are from the same population, are intermediate, indicating sperm mixing. The AB_B crosses, in which the first male is heteropopulation and the second male is conpopulation, have significantly higher P2. Finally, the AB_A crosses, with a conpopulation first male and heteropopulation second male, show low P2.

Figure 4-2. Expected P2 (light gray) and observed P2 (dark gray with hash lines) by cross for the GL (A) and GS crosses (B). Expected P2 within populations was assumed to be 0.5 to represent sperm mixing. This expectation is designated with a solid reference line in the figures. For crosses with males from different populations, expected P2 was calculated as the expected proportion of sperm transferred by the second male out of the total number of sperm transferred by both males. Crosses are described as Male1Male2_Female. If the two bars are similar, then sperm transfer is responsible for apparent conspecific sperm precedence. If observed P2 (dark gray) is higher than expected P2 (light gray), then the second male has a sperm competitive advantage. If the opposite is true, the second male is at a competitive disadvantage as a result of sperm competition.

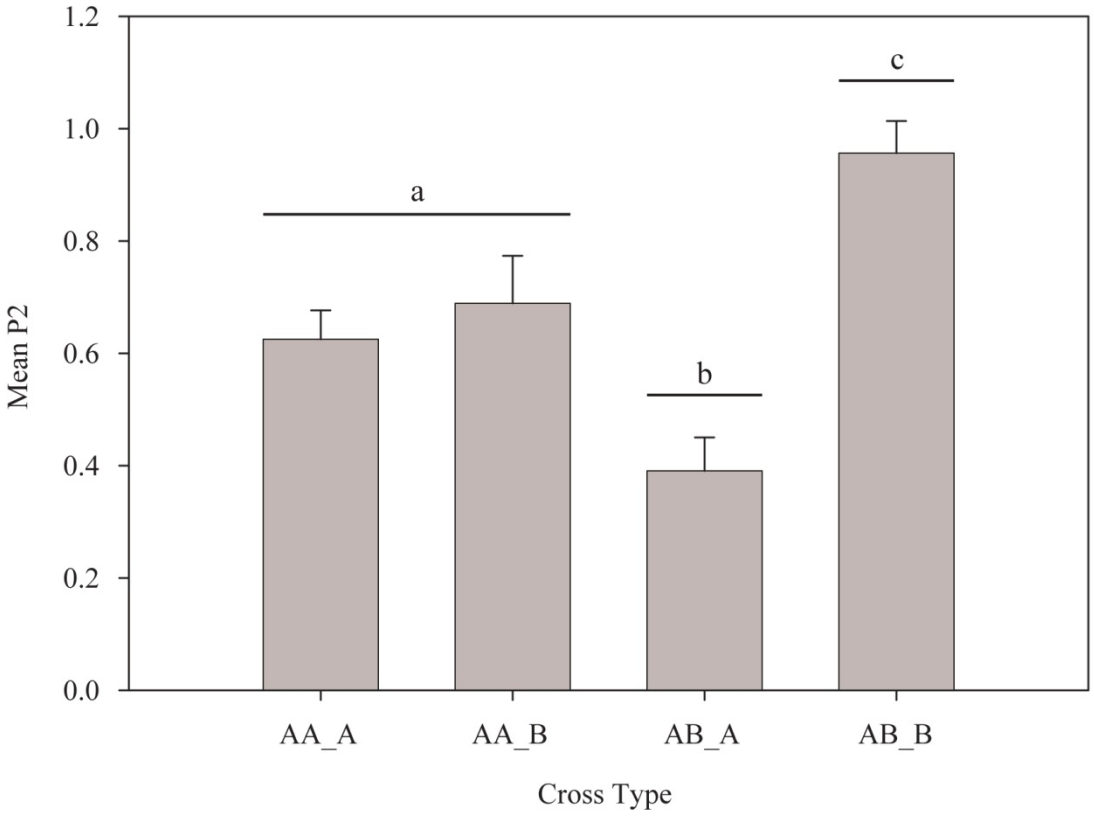


Figure 4-1

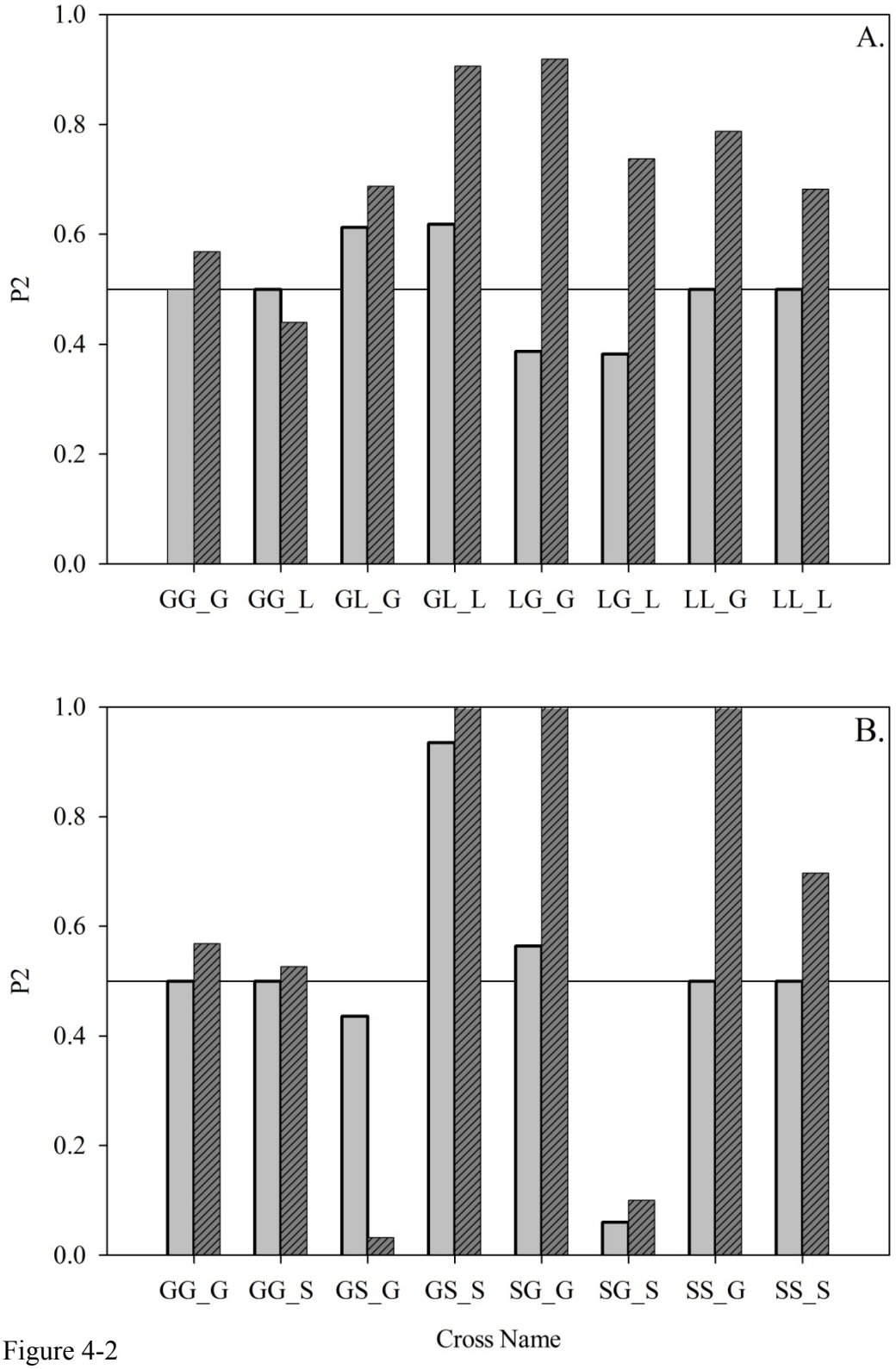

A.
P2
1.0
0.8
0.6
0.4
0.2
0.0
GG_G
GG_L
GL_G
GL_L
LG_G
LG_L
LL_G
LL_L
B.
P2
1.0
0.8
0.6
0.4
0.2
0.0
GG_G
GG_S
GS_G
GS_S
SG_G
SG_S
SS_G
SS_S
Cross Name

Figure 4-2

Chapter 5: General Discussion

One of the primary goals of speciation research is to understand the processes contributing to reproductive isolation among closely related populations which are in the process of diversification (Coyne and Orr 2004). In the preceding studies, I describe previously unidentified barriers to gene flow among closely related populations of the stalk-eyed fly species *Teleopsis*. Through these experiments, I was able to gain some insight into the mechanisms underlying these barriers. In the first study (chapter 2), I identified divergence in postcopulatory traits among populations and concluded that diversifying selection was the most likely explanation for the observed evolutionary divergence. In the second and third studies (chapters 3 and 4) I found evidence of non-competitive and competitive gametic isolation among allopatric populations of *Teleopsis* stalk-eyed flies. Several interesting future avenues of research emerge from these results.

One compelling avenue for future research would be to examine whether there is a connection between the morphological divergence observed in chapter 2 and the presence of gametic isolation observed in chapters 3 and 4. For example, evidence from a range of taxa demonstrates that sperm length affects fertilization success and sperm competition outcome within species (Garcia-Gonzalez and Simmons 2007b; Schulte-Hostedde and Millar 2004) and many have speculated that such divergence has the potential to drive speciation (Coyne and Orr 2004; Miller and Pitnick 2002). In stalk-eyed flies, we now have evidence of divergence of sperm length and sperm storage organ size among populations and of correlated evolution among these traits

(chapter 2). We also know that in crosses between these populations, sperm are less likely to reach the site of fertilization (chapter 3) and that heteropopulation sperm are generally at a competitive disadvantage (chapter 4). Sperm length and sperm storage organ morphology may play a causal role in these reproductive incompatibilities.

Another interesting future study would be an examination of relative rates of accumulation of reproductive isolating barriers among these populations of *Teleopsis* stalk-eyed flies. As described in chapter 4, there is now data on every level of reproductive isolation among these populations except ecological reproductive isolation. (A study of ecological speciation, which is driven by divergent natural selection among populations, would also be of interest). These data provide the rare opportunity to assess the evolution of various modes of reproductive isolation with phylogenetic replication.

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