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IRVINE

Experimental Tests of Speciation Mechanisms in *Drosophila melanogaster*

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biological Sciences

by

Larry Gonzaga Cabral

Dissertation Committee:
Professor Michael R. Rose, Chair
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DEDICATION

To

My wife Carrie and daughter Julia, for their love and support.

My sister Leslie, for her help and understanding.

My parents Maria and Luis, for teaching me the value of hard work.

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ABSTRACT OF THE DISSERTATION

Experimental Tests of Speciation Mechanisms in
Drosophila melanogaster

By

Larry Gonzaga Cabral

Doctor of Philosophy in Biological Sciences

University of California, Irvine, 2015

Professor Michael R. Rose, Chair

Since Darwin published the *Origin of Species*, biologists have contended that divergent natural selection is a common force in creating reproductive isolation. Others have disagreed, arguing for other evolutionary mechanisms instead. We used replicate outbred populations of *Drosophila melanogaster* that have been experimentally evolved for hundreds of generations under contrasting as well as parallel selection regimes, in order to test the importance of divergent selection compared to other evolutionary mechanisms in initiating reproductive isolation between allopatric populations. There is extensive genome-wide differentiation both between and within these groups of populations, according to analysis of their single nucleotide polymorphisms (SNP). Parental populations and their crosses were phenotypically assayed for the following characters: (1) mate-choice, (2) mortality, (3) fecundity, and (4) developmental success. In Chapter 1 we provide a literature review of the theories of speciation. We make a distinction between Darwin's theory of ecological speciation and the "null" theories of speciation in which ecological differentiation plays no role in the evolution of reproductive isolation. In Chapter 2 we tested the null theory of speciation by conducting within treatment crosses of three

groups of *Drosophila melanogaster* populations. We found only four population crosses out of 15 that demonstrated at most modest evidence for the null theory of speciation. In Chapter 3 we tested the ecological speciation hypothesis by conducting between treatment crosses of two groups *D. melanogaster* populations that have adapted to different selection regimes. We found strong evidence for the role of ecologically divergent selection among all population crosses in producing incipient reproductive isolation. In Chapter 4 we tested for the interaction between ecological selection and other evolutionary mechanisms in speciation. We did not find a statistically greater signal of reproductive isolation when the populations involved in any of these crosses had a greater time since their last shared common ancestor. This result suggests that there is no detectable interaction of time and selective differentiation in initiating reproductive isolation. Overall, our conclusion is that differences in selection regime have greater relative importance than evolutionary time in fostering reproductive isolation between allopatric populations.

CHAPTER 1

Introduction: Theories of Speciation

Abstract

The species concept has been the subject of debate amongst philosophers, naturalists, taxonomists, and eventually biologists since the time of Aristotle. From the Middle Ages to 1800, this debate was often framed in theological terms. In the early 1800s, geologists noted that fossils resembling the skeletons of modern species were rare in deep geological formations, suggesting that some species must have arisen after the events in Genesis. Thus the origin of species, or "speciation," became of interest to biologists. In 1859, Charles Darwin argued for evolutionary adaptation by natural selection as a mechanism for speciation, although much was left out of his pioneering discussion. During the 20th Century, Darwin's original model was developed into the ecological theory of speciation. But some evolutionary biologists have developed a very different view from Darwin's, instead proposing speciation hypotheses that feature little role for ecological adaptation. Thus the role that selectively-established differentiation of populations plays in first creating reproductive isolation remains contentious. This scientific issue is the central focus of the present doctoral thesis.

LITERATURE REVIEW

Introduction

> No one ought to feel surprise at much remaining as yet unexplained in regard to the origin of species and varieties, if he make due allowance for our profound ignorance in regard to the mutual relations of the many beings which live around us (Darwin 1859).

More than 150 years after the publication of Charles Darwin's 1859 *Origin of Species,* we still don't have a reasonable understanding of the details concerning how genetic, ecological, and evolutionary factors interact to create species. At the center of this failure of understanding is our lack of knowledge concerning the relative importance of natural selection versus other evolutionary genetic mechanisms in fostering initial reproductive isolation. It is this last question which is the central theme of this doctoral thesis.

The Species Concept

Aristotle

We start with the question of what is a species. The term "species" predates Darwin's concept of speciation by two thousand years. The first Western academic figure to develop a taxonomic system for living things was Aristotle (385-322 BCE). Further, at the foundation of his taxonomic system was the idea that living things are in fact delimitable into well-defined species that do not transmogrify into one another (Mayr 1982, pp. 149-154). Sometime after the death of his mentor Plato, during his first period of exile from Athens, Aristotle made his way to the Greek island of Lesbos around 347 BCE. During his time at Lesbos, Aristotle became

interested in zoology; he began compiling information about animals from breeders, merchants, as well as his own crude experiments (vid. Leroi, 2014). As David Hull (1988, pp. 75-77) notes, we may quibble over whether Aristotle was a scientist in a contemporary sense, but that he behaved as scientifically as any other biologist before 1800 cannot be questioned.

Aristotle's classification system grouped together animals with similar characters into a *genos* (race or stock) and then distinguished the *eidos* (form or kind) within the *genos*. This method came to be known in the Middle Ages as *per genus et differentiam*-"by the general type (genus) and the particular difference (species)" (Wilkins 2009, p.17). Aristotle ranked organisms on a linear "ladder of life", ordered according to complexity of structure and reproduction, with higher organisms having greater mobility (vid. Lovejoy 1936). But for our purpose, the most important feature of Aristotle's taxonomy was that his species were "fixed" in the sense that they could not transform into other species and thereby move up his ladder of life.

Medieval and Renaissance Philosophies of Biology

Aristotle's ladder of life was taken up by natural philosophers during the Scholastic period (1100 to 1700 CE) as part of a rigid hierarchal structure for all forms of matter and life, both natural and supernatural: "the great chain of being." Like Aristotle's ladder of life, the great chain of being was organized in a linear fashion in terms of a supposed "perfection of form," with minerals at the bottom, then plants, then animals, then humans, with angels and God above humans. Like Aristotle's ladder of life, the great chain of being presumed fixity, with species unable to transition or transform from one rank to another.

Medieval thought added additional hypotheses to Aristotle's fixity of species. One was the principle of continuity, that all forms of mater and life could be organized in a continuous

linear fashion, without any gaps between species. This idea derived from the medieval philosophical inference that gaps would indicate an incomplete creation, which could be interpreted as an imperfection (vid. Lovejoy 1936). Because God was supposed to be perfect, so must be his Creation. The principle of plentitude, the idea that all forms of life that could exist therefore *must* exist, was a logical extension of the notion of a perfect continuum. In other words, if you could imagine an intermediate form between two species, that intermediate must exist somewhere in the world. From ideas like this, medieval thought about biological species naturally concluded that extant species cannot evolve into new species, as the overall structure of the great chain of being was assumed to be fixed. Thus, speciation could not occur, as all species that could exist already exist, according to medieval Western biology.

Extinction was impossible in this system, at least by strictly natural processes. God could eliminate species by divine fiat, and human actions might remove species from specific parts of the world. But no natural process of extinction was allowed (Bowler 1989).

Evidently, ideas of this kind established a Western creationist biological orthodoxy very different from *both* the ill-defined transformations of one life-form into another allowed in other pre-modern theories of biology, such as those of Taoism (vid. Needham 1956), *and* any type of evolutionary hypothesis.

The great chain of being provided a crude taxonomic hierarchy, but didn't provide a definition for species. Creationist interpretation of the nature of *species* was heavily influenced by philosophical school of Essentialism (Mayr 1982, p. 256). Essentialists believe that, like all matter, species are characterized by an unchanging essence and separated from all other species by a sharp discontinuity. Essentialism assumes species diversity is the manifestation of a number of unchanging characteristics or qualities called *universals* (Hull 1976). All individuals that

belong to the same species share the same essence. Variation thus is the result of imperfect manifestations of the essence, on this view (vid. Wilkins 2009).

In practice the essentialist concept of species created more confusion than clarity. Comparing two individuals that share the same character is simple enough. However, when confronted with age-dependent characters, sexual dimorphism, or any kind of polymorphism, this line of thinking often broke down (Mayr 1982, p. 257). Morphological characters alone weren't enough to determine shared essence. Another criterion was needed.

Among the leading biologists of the Renaissance was John Ray (1627-1705), who provided foundations for the study of biology within England. Among many other notable contributions, Ray provided a biological definition of living species in his 1686 *History of plants:*

> ... no surer criterion for determining species has occurred to me than the distinguishing features that perpetuate themselves in propagation from seed. Thus, no matter what variations occur in the individuals or the species, if they spring from the seed of one and the same plant, they are accidental variations and not such as to distinguish a species... Animals likewise that differ specifically preserve their distinct species permanently; one species never springs from the seed of another nor vice versa (quoted in Mayr 1982, p.256).

Ray's definition was practical, but still emphasized the concept of a shared essence between species. The popularity of Ray's definition with subsequent generations of naturalists was in part due to how well it fit with the creationist dogma (Mary 1982, p. 257).

Linnaeus, Buffon, and Biology in the 18th Century

A great admirer of John Ray was Carl Linnaeus (1707–1778), who laid the foundations for the modern binomial species-naming scheme. Linnaeus perception of species was heavily influenced by his religious beliefs. In 1751, in the *Philosophia Botanica* (para 157), he writes:

> There are as many species as the infinite being created diverse forms in the beginning, which, following the laws of generation, produced as many other but always as similar to them: Therefore there are as many species as we have different structures before us today (quoted in Mayr 1982, p.258).

He later changed his views on the completeness of God's creation in response to a number of observations of new species created through hybridization (Wilkins 2009, p. 73). At first he thought that these hybrids represented intermediates in the great chain of being that should have existed as a distinct lineage, rather than the product of two others. But later he abandoned the initial definition of species that is quoted above, concluding instead that only genera had been created by God and that species might be the result of hybridization events within genera (Mayr 1982 p. 259).

Georges-Louis Leclerc, Comte de Buffon (1707–1788), was a French naturalist and mathematician. His works influenced the next two generations of naturalists, including Jean-Baptiste Lamarck and Georges Cuvier. Buffon's thoughts regarding species changed over the course of his lifetime. He has the distinction of being the first to make reproductive isolation the test to determine if two organisms are the same species.

> We should regard two animals as belonging to the same species if, by means of copulation, they can perpetuate themselves and persevere the likeness of the species: and we should regard them as belonging to different species if they are incapable of producing progeny by the same means (Hist. Nat., II: 10; quoted in Lovejoy 1959: 93f).

Mayr (1982) pointed out that Buffon had gone a long way toward introducing the biological species concept. But by considering species as constant and invariable, Buffon still adhered to the essentialist species concept.

Lamarck versus Lyell: The problem of the origin of species before 1859

Jean-Baptiste Pierre Antoine de Monet, Chevalier de Lamarck (1744 –1829), was a French biologist and the first proponent of the idea that evolution proceeded without divine intervention. Lamarck's treated each species as a single lineage that originated from a spontaneous generation event. Lamarck believed that organisms were not fixed in their current form and thus it was futile to argue over definitions of species (Wilkins 2009, pp. 104-108).

> Thus, among living bodies, nature, as I have already said, definitely contains nothing but individuals which succeed one another by reproduction and spring from one another; but the species among them have only a relative constancy and are only invariable temporarily (*Zoological Philiosophy* p. 44; quoted in Wilkins p. 105).

Despite Lamarck's dissenting view, in the early 19th century the Western academic consensus was that taxonomic species were genuinely distinct. But with every new fossil discovery of a species that evidently no longer existed, the idea that species have gone extinct during Earth's history could no longer be ignored. Furthermore, since modern species usually weren't found as fossils in geologically deep formations, some biologists in the early 19th Century were open to the view that some species must have arisen after the events in Genesis.

The problem was how species originated. Among those who made this problem prominent for European scientists was Sir Charles Lyell FRS (1797–1875), the leader of the British geologists during his career. Not incidentally, Lyell was an avid critic of Lamarck's hypothesis of evolution; he and Cuvier were the leading critics of Lamarck for the thirty years between Lamarck's death in 1829 until 1859. Thus, while Lyell was clear in pointing to the problem of the origin of species, he was even more trenchant in rejecting the Lamarckian evolutionary solution to the problem.

Darwin's views on the species question

Charles Darwin's views on the species concept shifted between his voyage on S.S. Beagle in 1831 and the publication of *Origin of Species* in 1859. At first he was quite comfortable with reproductive isolation as a test for species (all notebook quotations from Wilkins 2009, pp. 131-132).

> The dislike of two species to each other is evidently an instinct; & this prevents breeding (Notebook B, p.197).
>
> My definition of species has nothing to do with hybridity, is simply, an instinctive impulse to keep separate, which no doubt be overcome, but until it is these animals are distinct species (Notebook C, p.161).
>
> If they [systematists] give up infertility in largest sense as test of species-they must deny species which is absurd (Notebook E, p.24).

But by the time of the *Origin*, he seems to have taken the position that species are meaningless. Why Darwin changed his mind is debatable. Mayr (1982) believes Darwin was increasingly influenced by botanists such as William Herbert, who held the opinion that there are "no real or natural line of difference between species and permanent or discernible variety". An often quoted passage from the *Origin* makes a similar claim.

> From these remarks it will be seen that I look at the term species as one arbitrarily given, for the sake of convenience, to a set of individuals closely resembling each other, and that it does not essentially differ from the term variety, which is given to less distinct and more fluctuating forms. The term variety, again, in comparison with mere individual differences, is also applied arbitrarily, for convenience' sake (Darwin 1859, p. 42).

Darwin took Lamarck's position that evolution made the issue of defining species pointless, because their essence changes over time. " When the views entertained in this volume… are generally admitted…systematists… will not be incessantly haunted by the shadowy doubt whether this or that form be in essence a species. This I feel sure, and I speak

after experience, will be of no slight relief" (Darwin 1859 p. 484). Darwin never again attempted to define species after the publication of the *Origin* (Mayr 1982 p. 269).

Dobzhansky and Mayr

In 1935 Theodosius Dobzhansky, a Russian geneticist who worked in T.H. Morgan's Columbia University lab, published the paper, "A critique of the species concept in biology." He understood that the key to defining species was dependent upon understanding how a continuous process –evolution- could produce genetically discrete groups (Coyne and Orr 2004, p. 2). Dobzhansky proposed quantifying specific rank on the existence of reproductive isolating mechanisms which he defined as "any agent that hinders the interbreeding of groups of individuals…The isolating mechanisms may be divided into two large categories, the geographical and the physiological" (Dobzhansky 1937, p. 230). He defined a species as "…a group of individuals fully fertile inter se, but barred from interbreeding with other similar groups by its physiological properties" (Dobzhansky 1935, p. 353).

Ernst Walter Mayr (1904 –2005) was one of the 20th century's leading evolutionary biologists and a historian of science. He, as well as Theodosius Dobzhansky, contributed to the conceptual revolution that was the so-called "Evolutionary Synthesis." Mayr is usually given the lion's share of credit for defining the biological species concept, as follows: "A species is a reproductive community of populations (reproductively isolated from others) that occupies a specific niche in nature" (Mayr 1982, p. 273).

According to Mayr, the shift from an essentialist to a biological species concept required a change from thinking of species as "types" to envisioning them as populations. Furthermore, like Buffon, Mayr argued that reproductive compatibility should be the key test to determine

whether two populations belong to the same species. In this sense, Mayr's view was that species should not be characterized in terms of any "intrinsic" properties, but instead in terms of their reproductive compatibility with other co-existing species (Mayr 1982, p. 272).

There are 22 different species concepts in use in modern scientific literature (Mayden 1997). These species concepts vary considerably in their focus, depth , and applicability to various taxa. For the work presented in this dissertation, we will be using the Mayr "biological species concept" to define reproductive isolation.

Evolutionary Theories of Speciation, From Darwin to Mayr

Evolutionary history has two major features: (1) the branching of a lineage into two descendant lines, called *cladogenesis;* and (2) evolutionary dynamics of biological characters within species, called *anagenesis*. The taxonomic diversity of organisms is the consequence of cladogenesis, the branching of lineages. With the multiplication of lineages, which then evolve by anagenesis, the material diversity of life then increases. Each cladogenic branching point in the phylogenetic tree of life is necessarily a speciation event.

Modes of Speciation

The evolution of reproductive barriers to gene flow may occur under three kinds of geographic settings (Futuyma 2009, p. 472). Allopatric speciation is the evolution of reproductive barriers in populations that are prevented from exchanging genes by a geographic barrier. With parapatric speciation, populations are spatially distinct but adjacent, possible permitting some gene flow. Sympatric speciation is the evolution of reproductive isolation

within a single population, where there are no geographic barriers to restrict gene flow between individuals. Here the focus will be the evolution of reproductive isolation under allopatry.

Ecological mechanisms of speciation before Mayr

Implicit in Darwin's (1859) original theory of evolution by natural selection was the hypothesis that phenotypic divergence produced by natural selection would in turn lead to speciation, speciation being crudely conceived by Darwin as the process whereby evolutionary lineages become separate. Darwin initially thought speciation was driven by geographic isolation, but by the time of publication of the *Origin* his opinion changed in favor of selection against intermediates and hybrids. Naturalists, like Moritz Wagner (1889), Karl Jardon (1896), and David Starr Jordan (1905; 1908) held the opposite view, that geographic isolation had greater influence on the evolution of reproductive isolation (Mayr, 1982 pp. 561-566). However, they did agree with Darwin that natural selection was the most important force in speciation.

Mayr's model of allopatric speciation with ecological differentiation

In his book *Systematics and the Origin of Species* (1942), Mayr codified the biological species concept and proposed allopatric speciation as a mechanism for how multiple species could evolve from a single ancestor. He wrote that when populations within a species become isolated by geography, resource availability, mate choice, or other means, they may start to differentiate from other populations through natural selection, and over time they may evolve into new species.

Modern Theories of Ecological Speciation

In recent years, however, a more specific Darwinian view of speciation has been brought to the fore by Schluter (e.g. 2009), Via (e.g. 2009) and others (Funk 1998; Rundle and Nosil 2005; reviewed in Coyne and Orr 2004). Here we will refer to this view, as its proponents often do, as the "ecological speciation hypothesis." This hypothesis proposes that the evolution of reproductive isolation between populations depends on divergent phenotypic adaptation in response specifically to natural selection arising from differences between environments. This in turn implies that reproductive isolation should be more likely to evolve between allopatric populations that have significantly adapted to different environments, compared to allopatric populations that are well-adapted to similar environments.

Literature supporting the importance of ecological speciation

Evidence for the ecological speciation hypothesis has come from studies employing a "top-down" approach. Broken down into steps, this top-down approach involves (i) identifying the phenotypic traits under divergent selection, (ii) those traits associated with reproductive isolation, and (iii) the genes underlying traits and reproductive isolation (Schluter 2009). Step (iii) is the most challenging, but is key to understanding exactly how selection led to reproductive isolation.

It has been claimed that the ecological speciation hypothesis is supported by cases involving both prezygotic and postzygotic reproductive isolation. Ecological speciation has been inferred from instances of assortative mating involving body size and coloration in fish (McKinnon et al. 2004), beak size in birds (Podos 2001), pollinator preferences (Ramsey et al.

2003), and variation in flowering time (Lowry et al. 2008b). Ecological speciation has also been inferred from instances of unfit hybrids arising from both disrupted mimicry in butterflies (Jiggins et al. 2001; Naisbit et al. 2001) and intermediate migration patterns in birds (Helbig 1991).

Experimental evolution projects testing ecological speciation theory

Evidence supporting the hypothesis that ecological speciation occurs includes parallel speciation, where greater reproductive isolation repeatedly evolves between independent populations adapting to contrasting environments than between independent populations adapting to similar environments (reviewed in Rice and Hostert 1993; Schluter and Nagel 1995). In these experiments populations have been divergently selected for traits such as geotaxis (Hurd and Eisenberg 1975), temperature (Kilias et al.1980), caloric intake (Dodd 1989), and development time (Miyatake and Shimizu 1999). In each study, significant premating isolation was found between populations from contrasting environments, but no isolation evolved between populations that had been subjected to the same environment (Coyne and Orr 2004 p.88-89).

Null Theories of Speciation

The idea of speciation without any ecologically relevant selection

A number of 20th Century biologists developed a very different view from Darwin's about the role of natural selection in speciation. This alternative set of speciation hypotheses proposes that divergent adaptation to different environmental conditions during allopatry plays no role in fostering reproductive isolation (e.g. Rose and Doolittle 1983). These "non-

ecological" hypotheses imply that under identical selective pressures, given enough time, reproductive isolation will evolve. There are many such "non-ecological" speciation hypotheses, too many to be usefully reviewed here. However, we should be clear that this category includes the "mutation-order" speciation scenario (Mani and Clarke 1990), speciation by genetic drift (Lande 1981), and polyploidy speciation (Winge 1917). Recent synoptic reviews of such speciation scenarios have been provided by Schluter (2001), Turelli et al. (2001), Coyne and Orr (2004), Schulter (2009), and Sobel et al. (2009). Here we refer to all such hypotheses as collectively constituting a "null" theory for speciation, in order to clearly differentiate them from those which follow Darwin's lead in emphasizing functional differentiation brought about by selection in different environments, the collectivity we label ecological speciation theory. As will be shown below, grouping non-ecological hypotheses as not only is this a useful theoretical distinction (vid. Schluter 2000, 2001; Rundle and Nosil 2005; Schluter 2009, Nosil et al. 2009), it is also an experimentally practical dichotomy.

Pre-modern ideas of null speciation.

The Mutationists were a group of anti-Darwinian biologists who arose after the rediscovery in 1900 of Mendel's work (vid. Provine, 1971). Rejecting Darwin's claim that speciation was gradual and driven by natural selection, Mutationists like De Vries (1906), Bateson (1922), and Goldschmidt (1940) argued that speciation involves non-adaptive and macromutational leaps (Coyne and Orr 2004, p.2). Richard Goldschmidt proposed that species evolved through chromosomal "re-patterning" to form "hopeful monsters" (Wilkins 2009, p. 188).

Another null speciation concept is the mutation-order speciation hypothesis, which can be defined as the evolution of reproductive isolation by the fixation of different advantageous

mutations in separate populations experiencing similar selection pressures (Schluter 2009). Reproductive isolation is hypothesized to arise between populations that evolve different genetic solutions to the same selective pressures (Mani and Clarke 1990). The same alleles would have been favored in both populations, but divergence occurs because by chance the populations do not acquire the same mutations or fix them in the same order. Divergence is stochastic, but the process involves selection, and thus is distinct from genetic drift alone (Mani and Clarke 1990).

Null genomic theories of speciation

Despite recent advances in next generation sequencing, there is still a lack of understanding of (1) the genetic elements that underlie reproductive isolation, and (2) how these elements are organized in the genomes of diverging populations (Feder et al. 2012). Speciation could, conceivably, arise from inherited elements that are not conventional genes. For example postzygotic isolation could be caused by activation of transposable elements in hybrids. *Drosophila melanogaster* have recently undergone a global invasion of the P transposable element, starting with North American populations in the 1950s. When females from laboratory stocks that lack P elements are crossed with wild-type males which have P elements, the progeny display impaired fertility under some conditions, due to very high rates of transposition in the germ line. If allopatric populations undergo multiple, but different, invasions of transposable elements, then it is plausible to suppose that hybrids could suffer massive genomic disruption from extensive, unregulated transposition (Rose and Doolittle 1983; Krieber and Rose 1986).

An alternative genomic mechanism for reproductive isolation is one involving structural genome evolution. If one genome becomes very different in structure, hybrids may be subject to a failure of the genetic mechanism, in both gene transmission and gene expression. The Chinese

and Indian muntjac, two species of a type of small deer, illustrate this pattern. The Chinese species has 46 chromosomes, while the outwardly similar Indian species has 6 chromosomes. During allopatry, it appears, the Indian species has undergone numerous chromosome fusions. Hybrids between the two can be made, but they are infertile, due to incompatibility of the two genomes.

Experimental evolution projects testing null speciation theories

Sexual selection may cause divergence if reproductive isolation evolves by the fixation of advantageous mutations in different populations that are cultured under similar selection regimes (Nosil 2012). Long et al. (2006) performed reciprocal crosses between the "B" populations of Rose (1984), which had been maintained under identical conditions for 637 generations, at the time of their experiments. They found that seven of the 30 crosses with 'foreign' mates resulted in significant reductions in female components of fitness, whereas two resulted in significant increases in female components of fitness, compared to matings of individuals from the same population.

Sexual conflict can cause divergence in populations undergoing parallel selection in mating preferences or in gametic interactions that affect fertilization success (Rice 1998, Gavrilets 2000, 2004, Arnqvist and Rowe 2005, Gavrilets and Hayashi 2005, Sauer and Hausdorf 2009). Martin and Hosken (2003) cultured replicate populations of the dung fly *Sepsis cynipsea* under three rearing conditions: high-population density, low-population density, and monogamy. The rationale for the experimental design was the greater the number of potential mating partners, the greater the intensity of sexual conflict. They found reproductive isolation

evolved between replicate populations cultured with higher population density to a greater degree than small populations. This is a particularly good example of this speciation scenario.

Interaction Between Ecological Selection and other Evolutionary Mechanisms In Speciation

Darwin repeatedly emphasized that natural selection acts over long periods of time (Zimmer 2006; Reznick 2012). With evolution on a sufficiently long time-scale, it is likely that allopatric populations will undergo periods of parallel selection as well as periods in which ecological differences establish contrasting selection regimes. This raises the question whether longer-term evolution could produce reproductive isolation as a result of both ecological divergence and null sources of differentiation. For the sake of brevity, this type of scenario we will call the "Interaction" speciation hypothesis.

For example, it is conceivable that two allopatric populations could initially be subjected to very different ecological conditions, selection producing rapid functional differentiation between them. This functional differentiation could then stabilize, with some hundreds of generations of stable ecologically-defined selection following. An ecological speciation scenario would have reproductive isolation arise solely as a result of the initial period of rapid functional evolution. A null speciation scenario would involve reproductive isolation arising regardless of the period of functional differentiation; reproductive isolation would be favored simply as a result of the passage of evolutionary time. An *Interaction speciation scenario* would be one in which *both* are required, (i) the evolution of functional differentiation due to ecological differences and (ii) accumulation of genomic differentiation that is not related to functional differentiation, with protracted periods of parallel evolution in allopatry.

Testing the Alternative Theories of Speciation

It is easy to think up possible speciation scenarios. The harder problem is testing them in a definitive manner.

The central theme of this doctoral thesis is the relative importance of divergent natural selection due to differences in environments versus other evolutionary genetic mechanisms in fostering initial reproductive isolation. In order to define a precise antithesis to the former ecological speciation theory, the latter null theory for speciation can be defined as the group of mechanistic hypotheses for which divergent adaptation to different environments or culture regimes plays no role in fostering reproductive isolation. Two such broadly defined hypotheses for speciation might seem to afford few opportunities for critical hypothesis tests, but here we will argue that our laboratory can in fact perform strong-inference (vid. Platt 1966) comparisons of these two major theoretical alternatives for speciation.

Ideal System for Critical Tests of the Validity of Major Speciation Theories

In order to test the relative validity of the different major speciation theories, what is needed is a research system that has two different kinds of replicated populations. *First*, multiple replicated populations that have evolved in parallel in similar environments, such that only non-selective evolutionary mechanisms could produce incipient reproductive isolation. *Second*, multiple replicated populations that have undergone contrasting selection regimes that have led to the evolution of significant, functional, phenotypic divergence. In addition, there should be evidence that at least some of these populations are in the initial stages of evolving reproductive isolation of some type(s). It would be particularly advantageous if such an extensively

differentiated evolutionary system had few complications arising from historical accidents of differentiation that might make comparisons of evolving populations confounded by such accidents. For example, if all these populations were derived from a common ancestral population, many historically accidental evolutionary confounds could be precluded.

In such an ideal system, the central hypothesis test would be whether there is evidence for greater incipient reproductive isolation among populations that have phenotypically diverged due to selection, relative to the level of incipient reproductive isolation shown by populations that have been maintained under the same ecological regimes, but have evolved separately from each other. The particular advantage of this kind of hypothesis test is that it is not merely a hunt for corroborative or falsifying instances. Rather, if there are enough populations of each type, the hypothesis test can be formulated quantitatively as a well-defined comparison of relative magnitudes of reproductive isolation between that have phenotypically diverged as a result of selection and those that have not.

In our present experiments, we have been making crosses that give well-defined opportunities for the action of either null or ecological speciation mechanisms in the evolution of our laboratory populations. But the natural question that follows on from the hypothesis tests of those first two phases of our experimentation is whether or not there are significant interactions between these two potential contributors to reproductive incompatibility, as hypothesized in the Interaction speciation scenario defined here. Fortunately, our experimental evolution phylogeny of laboratory populations naturally allows us to readily combine wide disparities in *both* ancestry and phenotypic differentiation among crosses.

Our work will provide tests of both null and ecological speciation during the experimental evolution of incipient reproductive isolation. It will also provide quantitative measures of their relative significance as well as any synergism between them.

CHAPTER 2

Testing Null Speciation Theories

Abstract

Non-ecological explanations of speciation suggest that reproductive isolation can arise as result of divergence in allopatry without the action of ecologically established divergent selection. These non-ecological hypotheses imply that under identical selective pressures, given enough time, reproductive isolation will evolve even in the absence of ecologically divergent selection regimes. We refer to these hypotheses collectively as the null theory of speciation. We tested this theory using three groups of *Drosophila melanogaster* populations that share a common ancestor, each group having five replicate populations and a common selection regime. The three groups or "treatments" differ in development time (from egg to adult), one group highly accelerated, one intermediate, and one delayed. Both the highly accelerated and intermediate treatments have undergone approximately 800 generations of selection, twice the number of generations as the delayed treatment. Within-treatment population crosses were created and analyzed genetically for single nucleotide polymorphism (SNP) differentiation and phenotypically using four assays: 1) mate-choice, 2) mortality, 3) fecundity, and 4) development. Despite genetic as well as phenotypic differentiation among within-treatment populations, we have found of 15 population crosses; two cases of hybrid vigor and only four population crosses that demonstrated at most modest evidence for the null theory of speciation. Our conclusion is that the null theory of speciation does not survive this critical test.

Introduction

In the 20th Century, some biologists developed a very different view from Darwin's about the role of natural selection in speciation, proposing mechanistic speciation hypotheses featuring little or no role for ecological adaptation in the speciation process (e.g. Rose and Doolittle 1983). These "non-ecological" speciation hypotheses can be grouped into two categories: (1) mechanisms that do not involve organismal selection at all; and (2) mechanism that do involve organismal selection, but in which selection is not different among the environments of allopatric populations (vid. Nosil 2012). There are too many of these hypotheses to be usefully reviewed here, although we should be clear that the first group includes speciation by genetic drift (e.g. Lande 1981) and polyploidy speciation (e.g. Winge 1917), while the second includes the mutation-order hypothesis, which is defined as the evolution of reproductive isolation by the fixation of different advantageous mutations in separate populations experiencing similar selection pressures (Mani and Clarke 1990). Recent synoptic reviews of speciation scenarios have been provided by Schluter (2001), Turelli et al. (2001), Coyne and Orr (2004), Schluter (2009), Sobel et al. (2009), and Nosil 2012). Here we refer to all hypotheses that do not feature divergence as a result of organismal selection arising from ecological differences collectively as the "null" theory for speciation, in order to clearly differentiate all these ideas from those which follow Darwin's lead. As will be shown below, not only is this a useful theoretical distinction (vid. Schluter 2000, 2001; Rundle and Nosil 2005; Schluter 2009, Nosil et al. 2009), it is also an experimentally practical categorization.

Evidence supporting null speciation hypotheses is diverse, albeit indirect. Experimental evolution studies have suggested that uniform selection on isolated populations can lead to some

degree of reproductive incompatibility, such as reductions in the female component of fitness in *Drosophila* (e.g. Long et al. 2006) and *Sepsis* (Martin and Hosken 2003). More indirect evidence in support of null theories of speciation comes from instances in which reproductive isolation has seemingly evolved as a by-product of intragenomic conflict, such as cytoplasmic male sterility in plants (e.g. Fishman et al. 2008, Sambatti et al. 2008), and meiotic drive in *Drosophila* (e.g. Presgraves 2007a, b, Presgraves and Stephan 2007, Phadnis and Orr 2009, Tang and Presgraves 2009).

In order to define a precise antithesis to the ecological speciation theory, the null theory for speciation will be defined here as the group of mechanistic hypotheses for which divergent phenotypic adaptation to different environmental conditions during allopatry plays no role in fostering reproductive isolation. Thus if population size, mutation rates, et cetera are relatively uniform among a collection of isolated populations, we can ask *does reproductive incompatibility always arise as result of evolutionary divergence in allopatry* ***without*** *the action of ecologically established divergent selection*?

We ask specifically whether reproductive incompatibility *always* arise as result of evolutionary divergence in long-sustained allopatry without the action of ecologically established divergent selection, because it is impossible to test strongly a theory that suggests reproductive incompatibility *sometimes* arises under such conditions. Such "sometimes" theories are inherently not falsifiable. To be clear, the null theory does not suggest that reproductive isolation will arise only after short amount of time, such as a few generations. Rather the null theory suggests that under identical selective pressures, given enough generations, reproductive isolation will always evolve.

In order to address this over-arching question, what is needed is a research system that has multiple replicated sexual populations that have evolved in parallel in similar environments for *many* generations. This experiment must, however, all the action of any evolutionary mechanisms that don't involve divergent adaptation in response to different selection regimes. In addition, there should be evidence that at least some of these populations are in the initial stages of evolving reproductive isolation of some type(s). It would be particularly advantageous if such an extensively differentiated evolutionary system had few complications arising from historical accidents of differentiation that might make comparisons of evolving populations confounded by such accidents. For example, if all these populations were derived from a common ancestral population, at least some historically accidental evolutionary confounds could be precluded.

In our laboratory, we have such a research system. Our stock system is comprised of dozens of populations of *Drosophila melanogaster* divided among distinct selection treatments, with five or six replicate populations maintained for each selection regime (Rose et al. 2004). The estimated effective population size for each of these populations is about 1000 (Mueller et al. 2013).

For the 15 populations that we are particularly interested in for this Chapter, the selection treatments differ chiefly with respect to the length of their discrete generations: that is, the life-cycle from the egg-laying that starts one generation to the egg-laying that starts the next generation. Of particular importance for theories that require sufficient evolutionary time for their hypothesized mechanisms to act, these replicated treatment-groups constitute lineages that have been sustained for as much as 900 generations (see Figure 2.1).

Complementing the extensive phenotypic differentiation within our research system is evidence of incipient reproductive isolation among replicate populations from the B treatment

(Long et al., 2006). Long et al. (2006) performed reciprocal crosses between the six IV and B populations, which had been maintained under identical conditions for 637 generations at the time of their experiments. They found seven of the 30 crosses with 'foreign' mates resulted in significant reductions in female components of fitness, whereas two resulted in significant increases in female components of fitness, compared to local matings.

Here, we present data on incipient reproductive isolation in the three longest-standing treatments in our stock system: ACO, B, and CO. Within-treatment crosses were subjected to four assays to test the strength of prezygotic and postzygotic reproductive barriers: (1) mate-choice, (2) mortality, (3) fecundity, and (4) development rate.

Materials and Methods

Experimental Populations

Experimental evolutionary history: This study uses outbred lab populations of *Drosophila melanogaster* selected for different patterns of age-specific reproduction. All the flies used in this study ultimately originate from an ancestral "IV" population first collected from South Amherst, MA in 1975 by Phillip Ives (vid. (Rose 1984)), and then cultured in the lab using two-week discrete generations. These ancestral IV flies were subsequently used in February 1980 to create five "O" (old) replicate lines (Rose 1984). The IV flies were also used to found five additional "B" (baseline) populations in February, 1980, populations which have since been cultured using the same protocol as the IV line from which they were derived (see Figure 2.1).

Culture regimes: Over subsequent years, additional treatments were derived from the O populations using three distinct culture regimes: "A," "B," and "C" (see Figure 2.2). ***A*** *culture*

regime: the five ACO and five AO populations spend their first 9 days of life in 8-dram glass vials, and at day 10 are transferred to a Plexiglass "cage" in which they are given fresh food and allowed to oviposit for 24 hours. ***B** culture regime*: the five B and five BO populations spend 14 days in 8-dram vials, and are then allowed 1-2 hours in fresh vials to oviposit before adults are discarded. ***C** culture regime:* the five CO and five nCO populations develop in vials for 14 days prior to being transferred to Plexiglass cages. C flies are then given 48 hours to oviposit before eggs are collected on day 28. All populations are supplied with food made from cooked bananas, barley malt, yeast, corn syrup, and agar. The populations that spend time in cages are also supplied with live yeast on the medium surface prior to egg laying.

Test-Cross Experimental Design

Overall experimental structure: Three sets of within-treatment experiments were performed using the fifteen ACO, B, and CO populations. In effect, there were three separate sets of crosses performed, one for each selection regime. The three entire sets of crosses were repeated twice, with blocks approximately 6 months apart, which mitigated the impact of random environmental and handling effects on the results. There was a systematic experimental design difference between the two blocks. Single assay cohorts were used for each test-cross in block 1, while two assay cohorts were used for block 2. See Figure 2.3 for an overview.

Round-robin crossing system: Crosses within treatments were performed in a "round-robin" fashion: 1×2, 2×3, 3×4, 4×5, and 5×1. For each within-treatment cross, three types of flies were assayed: ancestor ("**a"**), cohabiting ("**c"**), and ("**F_1"**) hybrid. A ("**F_2**") hybrid was also used, but only for the development duration rate assay. Ancestor flies were obtained from crosses between males and females from a single ancestral population (e.g. all flies sampled from

ACO_1). Cohabiting flies have females from one ancestral population living with males of another ancestral population (e.g. ACO_1 females cohabiting with ACO_2 males). Hybrid flies are the F_1 offspring of a co-habiting population cross (e.g. all F_1 $ACO_1 \times ACO_2$ flies are true hybrids between population ACO_1 and ACO_2).

Rearing and sampling of assay cohorts: D. melanogaster cultures were initiated (day 0) in 25×95 mm vials containing 20 ml of banana/agar/yeast media at a density of 70 eggs per natal vial for each population test-cross.

In order to maintain regime-specific conditions throughout the experiment, special natal vials were created. These natal vials were made of two components: a 23×25mm cap and a 25×95 tube. The cap containing fly medium was inserted into the tube to create a vial of standard dimensions. After reaching the appropriate development stage, larvae would then climb the walls of the tube to pupate.

Virgin adults were collected using light CO_2 anesthesia as they eclosed from their pupal cases on day 8 for the ACO populations, day 9 for B populations, and day 10 for CO populations. Flies were sexed, crossed, and then placed into holding vials that consisted of a cap from the natal vials inserted into a clean tube. Population test-crosses were made by combining 25 virgin flies of each gender in the females' natal-capped holding vial, ten vials per population-cross, with 500 flies total for each such cross.

Adults were allowed to mate and freely interact in the females' natal vials until the normal culture day: day 10 for ACO and day 14 for B. The CO flies were transferred into cages at day 14, and then eggs were collected from those cages on days 26 to 28, from egg. These experimental procedures closely mimicked the normal culture regime experienced by these populations (see Figure. 2.2). Thus the assays performed in this study provide a reasonable

estimate of fitness under the culture conditions that each type of fly had experienced for hundreds of generations.

Overview of our analytical strategy

The analysis of our data is based on a specific scenario for the resumption of contact between members of our lab populations, conceived as a model system for incipient reproductive isolation between long-allopatric populations. As this scenario is somewhat complicated, we have provided both a schematic, shown in Figure 2.4, and the following verbal summary.

If two populations have long been divided by a major geographic barrier, the likelihood that a single migrant will challenge their reproductive isolation is remote. What we have in mind instead is the following steps.

Step One. A propagule from one geographical area migrates to the other geographical area, where this propagule consists of enough individuals so that it does not suffer from notable inbreeding depression. Note, however, that this initial migration is not assumed to immediately lead to hybridization. Thus the initial analytical question is how well this propagule group can survive under the selective conditions imposed in the geographical area to which it has newly migrated.

Step Two. Over some part of its range in its new geographical area, the migrant propagule group cohabits with the endemic population that has long undergone adaptation to that geographical area. During this phase of the process, matings may occur between endemic and propagule individuals specifically in the zone of cohabitation. At this point, *prezygotic* components of reproductive isolation come into play.

Step Three. As a result of hybridization events, some part of the geographical area constitutes a hybrid zone, in which hybrids and individuals with uncrossed parental genomes

constitute a mixed population. At this point, the relative fitness of hybrid progeny compared to parental genomes play an important evolutionary role. Their relative fitness can then be assayed with respect to *postzygotic* components of reproductive isolation.

Reproductive Isolation Assays

Our assays tested for both prezygotic and postzygotic reproductive isolation, comparing hybrids to uncrossed individuals for each assay. The prezygotic characters tested were (i) survival to the time of mating and (ii) mate choice. The postzygotic characters tested were (i) fecundity and (ii) development. Uncrossed and hybrid individuals are tested simultaneously by deriving the hybrids from the previous parental generation.

Life-history assays covered the entire range of ages during which any of the tested populations are maintained in our lab, from day 0 (from egg) to day 28 (from egg). This time period includes the longest duration that any adult fly is allowed to live in our present stock system, which no longer includes the O populations of Rose (1984). Selection-regime "focal" fitness was calculated from data collected specifically during the reproductive window of each treatment's generation cycle (as shown in Figure 2.2).

Mate choice assay: Two mate-choice tests were used: (1) a female from an endemic population was given the choice to mate with one of two suitors: a male from her own population and a male from a migrant population; and (2) same procedure as test (1), but with a migrant female as the choosing female instead of an endemic female. Each male was given colored yeast paste to ingest for identification purposes, with rotating combinations of colors among types of males. The flies were given two hours in which to mate. A successful mating event was scored when a male mounted a female for thirty seconds or more. Mate choice assays were conducted at

24 hours from eclosion using virgin flies. If females did not mate at all within the two hours, the experimental trial was discarded. Males were classified as (i) mated or not mated, (ii) marked or not marked. Sixty choice-assays were performed for each type of test, per block.

Adult survival assay: Flies from holding vials were transferred to population cages at each treatment's normal day of transfer out of their rearing vials: day 9 for ACO flies; day 14 for B flies; and day 14 for CO flies. Population cages were surveyed for dead flies before food plates were replaced each day. The dead flies were removed, then sexed, and their number recorded. Each cage housed 500 flies.

Net fecundity assay: Each assay cage contained a single Petri dish of food medium. Almost all of the eggs were laid in or on that Petri dish. During the adult survival assay, the Petri dish was removed from the cage daily. The removed Petri dish was rinsed with bleach solution in order to collect all the laid eggs onto a membrane placed within a Buchner funnel. The membrane was then placed on a flatbed scanner and photographed. The number of eggs laid was counted from this photographic image using ImageJ software. Net fecundity at a particular age is normally rendered as k_x ($k_x = l_x m_x$, where l_x is probability of survival to age x and m_x is fecundity at age x).

Development assay: For ACO and CO, 20 vials of ~70 eggs were collected from the population cages on the normal culture day, days 10 and 28 respectively. For the ACO and CO treatments a fraction of the number eggs that laid during focal fecundity were used for development. For the B population crosses, all eggs that were laid in ten vials during a two-hour period were used for the development assay. The number of eggs that developed into adults was recorded every 4 hours from day 8 to day 14, the last day on which adults are collected from vials. The number of adults that eclosed during the development duration assay was added to the

focal fecundity total. For B populations, development duration and focal fecundity are not mutually exclusive and thus the number of adults that emerge by day 14 is considered focal productivity and reported as k_x.

Reproductive Isolation Values

We quantified reproductive isolation between populations using a composite measure derived from the test-cross data. Our component measures of Reproductive Isolation Value (RIV) specify the strength of reproductive isolation inferred from each test-cross assay. This component index of reproductive isolation was calculated using the method proposed by Coyne and Orr (1989; 1997)

$$RIV_n = 1 - \frac{competitor}{maternal\,ancestor} \qquad (1)$$

where the subscript *n* refers to the specific character under study (e.g. mate choice). All these component indices of isolation reflect statistically significant differences between "competitor" (e.g. hybrid) and ancestral individuals in each test cross. RIV estimates are expected to vary between negative and positive values, where one is complete reproductive isolation. Scenarios in which hybridization is favored, as a consequence of disassortative mating or hybrid vigor, result in negative reproductive isolation values.

To calculate a composite measure of reproductive isolation, we used the method proposed by Ramsey et al. (2003): where the "life-history contribution" ("*LHC"*) of a component of reproductive isolation value (*RIV*) at stage *n* in the life history is calculated in the following manner:

$$LHC_1 = RIV_1, \qquad (2)$$

$$LHC_2 = RIV_2\left(1 - LHC_1\right), \text{ and} \qquad (3)$$

$$LHC_3 = RIV_3\left[1 - (LHC_1 + LHC_2)\right]. \qquad (4)$$

Generally: $$LHC_n = RIV_n\left(1 - \sum_{i=1}^{n-1} LHC_i\right). \qquad (5)$$

In this parameterization, a particular component reproductive barrier is taken to eliminate gene flow that has not already been prevented by previous components of reproductive isolation. To calculate total reproductive isolation in our study, four sequential life-history stage components were used: (1) focal fecundity of the ancestor populations; (2) mate choice between ancestor-population flies; (3) focal fecundity of the cohabiting flies; and (4) focal fecundity of the F_1 hybrids. For *m* components of reproductive isolation, total reproductive isolation (*T*) is

$$T = \sum_{i=1}^{m} LHC_i . \qquad (6)$$

As *T* reaches one, reproductive isolation becomes complete.

F_{ST} estimates: F_{ST} estimates were calculated at every single nucleotide polymorphism ("SNP") across the major chromosome arms to estimate genetic differentiation among within-treatment populations. To do this, SNPs were first called across 30 populations of the Rose stock system, including the 15 populations studied here as well as 15 other populations not studied here. SNPs were discarded if coverage in any of the populations was less than 20X or greater than 500X. We also required a minimum minor allele frequency of 2% across all 30 populations. Based on these criteria, ~1.13 million SNPs were identified across the major chromosome arms. A SNP table with major and minor allele counts for each SNP in each population was then generated.

SNP frequencies were taken directly from these counts and F_{ST} Estimates were obtained using the formula: $F_{ST} = (H_T - H_S)/H_T$ where H_T is heterozygosity calculated from the overall allele frequencies, and H_S is the average observed heterozygosity in each replicate population (Hedrick 2009). Estimates we made at every SNP across the major chromosome arms. This calculation was done in two ways: (i) for all replicate populations within a given treatment, and (ii) between specific replicate pairs with a treatment.

Data Analysis

Mate-choice data: The counts of males in each of the possible cells from this experiment were analyzed using a chi-square test. Marking was necessary to distinguish between males from different population crosses. However, since it may impact the females' preference, we controlled for this by rotating colors amongst types of males. Therefore, we were able to test whether mating status is independent of marking status in each experiment.

Survival and Fecundity data: We tested for differences in survival and fecundity due to population-cross status over relative and absolute ages using a linear mixed effects model. The observations consisted of fecundity at a particular age (*t*) but within a small age interval. These age intervals were chosen to span the ages such that all comparison populations still had live flies. Within each interval, survival or fecundity rates were modeled by a straight line and allowing population type (*j*= 1 (**a** 1), , 2 (**a** 2), , 3 (**c** 1), 4 (**c** 2), 5 ($\mathbf{F_1}$ 1), 6 ($\mathbf{F_1}$ 2)) to affect the intercept of that line but not the slope. However, slopes were allowed to vary between intervals. As with the other analyses, cages (*i*=1,…,12) were assumed to contribute random variation to these measures. With this notation, the survival (or fecundity) at age-*t*, interval-*k*, selection regime-*j* and population-*i*, is y_{ijkt} and is described by,

$$y_{ijkt} = \alpha + \beta_k + \delta_j\gamma_j + (\omega + \pi_k\delta_k)t + \delta_k\delta_j\mu_{jk} + c_i + \varepsilon_{ijkt} \qquad (7)$$

where $\delta_s = 0$ if s=1 and 1 otherwise and c_i, and ε_{ijkt} are independent standard normal random variables with variance σ_c^2 and σ_ε^2 respectively. The effects of selection on the intercept are assessed by considering the magnitude and variance of both γ_j and μ_{jk}.

Development data: Successful adult emergence prior to culture day was analyzed using log-linear hierarchical models. We numerically identify the classification variables as emergence-1, population cage-2, and vial-3 with block 1 and 2 done separately. If we model the counts in each cell as simply the sum of each log of the probabilities of each factor, the appropriate statistical model is C_1+C_2+C_3 . The model term C_{12} indicates the sum of a two-way interaction between mating status and marked status ($C_{1:2}$) as well as the separate factors C_1 and C_2, i.e. $C_{1:2}$+C_1+C_2. Models are tested by taking the difference of the likelihood ratio, or G^2 statistic (Bishop et al. 1975), of each model. This difference has a chi-squared distribution with degrees of freedom taken from the two models.

Results

Genomic assays of replicate population differentiation within selection regime.

A basic question in a study of this kind is whether or not there is any kind of genetic differentiation between replicate allopatric populations within selection regimes. After all, *if* there has been so much between-line migration that there is no genetic differentiation, *then* an experimental design like the present one would not adequately test the null hypotheses that have been proposed for speciation.

Fortunately, genome-wide sequencing studies have been performed with all 15 of the populations studied here (Graves et al., in prep.). Here we report for the first time estimates of F_{ST} values for the pairwise differentiation between the replicate populations of the three selection regimes we have used in this study (See Table 2.1). These F_{ST} values were calculated in two ways.

Firstly, the F_{ST} were calculated among all replicate populations within a selection regime. The three resulting values were 0.06, 0.062, and 0.04 for ACO, B, and CO populations, respectively. The first two values are comparable to those found in a study of differentiation among *Drosophila melanogaster* populations that had migrated to the East Coast of Australia (Kolaczkowski et al. 2011), about 0.112. Those colonizing populations undoubtedly went through a bottleneck on their way from Europe, Africa, or the Americas to the isolated continent of Australia. Since reaching the Eastern part of Australia, which provides abundant human and natural habitat suitable for *D. melanogaster,* the local populations must have expanded greatly in effective population size. But they are evidently less differentiated than comparable populations in Africa, where the species originated (Langley et al. 2012). However, the F_{ST} values among CO populations are not as high as those among the ACO and B populations. This is not surprising, from an evolutionary genetic standpoint, in view of the much smaller number of generations since the CO replicate populations last shared a common ancestor about 350 generations ago, compared to the more than 800 generations since the ACO and B populations last had a common ancestor.

Secondly, we also calculated F_{ST} values between pairs of replicate populations. Since we tested for reproductive isolation between pairs of replicate populations, using hybrids et cetera, their degree of relative molecular genetic differentiation was also of interest. For paired

populations, the F_{ST} values were much lower, as shown in Table 2.1. This can be understood as a function of the number of populations used in the F_{ST} calculation. The same procedure for SNP calling was done for both F_{ST} measures. However, sites that are polymorphic across the entire data set are not necessarily polymorphic in every paired comparison of two populations.

Overall, this is evidently an evolutionary genetic model system that features significant molecular genetic differentiation between replicate populations. Whatever accidental migration events might have occurred over the hundreds of generations of lab evolution involved in their creation, these replicate populations are in fact cases of parallel and at least somewhat independent evolution, rather than arbitrarily divided samples from a panmictic population.

Differentiation between evolved populations within selection regimes

Despite parallel selection regimes, the genome-wide differentiation particularly of the B and ACO populations, within their selection regimes, suggests the possibility of evolutionary genetic differentiation at functional sites within their genomes. That is, individual replicate populations that share a common selection regime, but have nonetheless differentiated for SNPs, could have responded to selection at different loci. At one extreme, this might have produced significant phenotypic differentiation among replicate lines, within selection treatments.

Because the experiments that we performed involved multiple measurements of some characters that did *not* depend on interactions with other populations, we have useful data for testing the hypothesis that parallel selection within selection regimes could nonetheless have produced phenotypic differentiation among replicate populations that share such selection regimes. This is tested in the analyses summarized in Tables 2.2-2.4.

These analyses were performed in two different ways, because Block 2 of the overall experimental design featured two complete sets of "round-robin" crosses, not the single set of

Block 1. Thus Block 2 effectively featured two "sub-blocks." This extreme imbalance of experimental design led us to analyze the data in two ways. First, we dropped the second sub-block from the data of Block 2, giving two blocks of data, in each of which the original replicate populations were measured twice for mortality rate, total fecundity, and focal fecundity. The results of that analysis are shown in Part A of Table 2.2, Part A of Table 2.3, and Part A of 2.4. Since that analysis yielded no significant results, we relaxed statistical stringency somewhat, and treated the two sub-blocks of Block 2 as fully independent Blocks, increasing the body of relevant data by a third. The results of that analysis are shown in Part B of Table 2.2, Part B of Table 2.3, and Part B of 2.4. In that analysis, we have three cases of statistical significance, with F values of 6.81, 7.12, and 12.33 making it over the $p < 0.01$ thresholds for significance. We repeat, however, that this statistical design isn't quite correct, as the "three-block" statistical analysis overestimates the power of our results.

For the characters of mortality rate, total fecundity, and focal fecundity, our opinion is that this analysis yields marginal evidence for differentiation of mortality rates and total fecundity among replicates within B selection regimes, as well was mortality among replicates within CO selection regimes. Therefore, the basic results of these tests indicate no more than the beginnings of functional differentiation among replicate populations, within selection regimes.

Overall hybrid vigor and hybrid inferiority effects within selection regimes

Even with modest functional differentiation of replicate populations within selection regimes, it is still conceivable that there has been functional convergence among the replicate populations because of hidden genetic heterogeneity. That is to say, it is possible that allele frequency changes at different loci could be responsible for producing convergent phenotypic results, among the replicate populations sharing a common selection regime. Indeed, this

possibility is one way to achieve reproductive isolation, if the different loci involved in this hypothetical scenario have adverse epistatic effects when they are differentiated, and then produce hybrid inferiority in crosses.

One way to test for such an effect would be assays of individual crosses between pairs of replicate populations within selection regimes. Those are the concern of the next sub-section of these Results.

A cruder test is to look for a general effect of crossing among replicate populations for the focal character closest to fitness. Specifically, even though these replicated populations are at most marginally differentiated for the key characters analyzed in the preceding sub-section, cryptic functional genetic differentiation could produce a general pattern of underdominance among their crosses. Such a pattern of underdominance would then foster reproductive isolation among the populations that share a selection regime, specifically if that underdominance impinges on the focal determination of fitness characteristic of that selection regime.

With this possibility in mind, we statistically analyzed the distribution of the following "hybrid vigor" value for our most appropriate "focal" fecundity ("FF"), our best surrogate for fitness itself under each of the three selection regimes:

Ancestral Population 1. (mean FF) + Ancestral Population 2. (mean FF)

Minus the sum of the two Reciprocal Cross FF values.

For strictly additive average patterns of inheritance, this value is expected to be zero, naturally. A signal of underdominance for this parameter arises when this value is sufficiently large and negative. With such a result, we would have suggestive evidence for hybrid incompatibility that would foster reproductive isolation.

The results of our statistical analysis of such potential underdominance are shown in Table 2.5. For the B populations, our results suggest an additive average pattern of inheritance for their focal fecundity character. On average B ancestral and hybrid populations laid about 14 egg per female (95% C.I. [-0.472, 0.472]). For the ACO (95% C.I. [2.30, 3.37]) and CO (95% C.I. [1.24, 4.01]) populations, averaged over all crosses, we find some evidence for directional dominance that makes the hybrid resemble the fitter parent, the opposite result from that expected with general underdominance. On average hybrids laid about 1 more egg per female than ancestral populations.

Reproductive isolation among specific population crosses within selection regimes

Given no general pattern of underdominance among replicate populations, within selection regimes, we next turn to an analysis of the full range of phenotypic data produced from individual between-population crosses.

It should be noted here that development wasn't incorporated in the invasion scenario. Under B-type regime conditions, development duration and focal fecundity are not mutually exclusive and thus the number of adults that emerge by day 14 is considered focal productivity and reported as k_x. For populations tested under A-type conditions the majority of the results varied significantly between blocks with no discernible pattern. Under C-regime conditions, no statistical differences in development were found (p =1.000).

ACO Treatment

Fecundity: Figure 2.5 shows the fecundity results for the individual crosses among ACO populations. Two types of population-crosses gave results that indicated statistically significant difference in fecundity. Specifically, the ACO_5 population had a lower fecundity than both the F_1

hybrid A_{51} ($p = 0.045$) and ACO_1 population ($p = 0.006$). The ACO_5 populations produced about 11 eggs per female, while the F_1 A_{51} population produced 16 eggs per female and ACO_1 population produced 17 eggs per female.

Development: Table 2.6 shows the analysis of developmental success for adult emergence at nine days among the individual population crosses. [A-type flies are given nine days to develop into adults before being transferred into cages.] The percent of adults that developed before and after day 9 were compared among ACO crosses. The majority of the results varied significantly between blocks with no discernible pattern. Only two pair-wise comparisons had results that were consistent between blocks. Hybrid F_1 A_{32} had a greater percentage of adult emergence than its maternal ancestor ACO_3 with $p = 0.011$ for block 1 and $p = 0.023$ for block 2. The ACO_5 vs. F_1 A_{51} comparison showed no significant difference in both blocks with a $p = 0.115$ and $p = 0.332$, respectively.

Mate-choice assay: Table 2.7 shows the analysis of the mate-choice results among ACO populations. None of the ACO females exhibited any statistically distinguishable mating preference. Initial analysis suggested at least one case of mate preference among most of the cohort pairs when specific combinations of color and male were used. However, this case of mate preference was obscured when the statistically significant effects of color was taken into account.

Reproductive isolation values: Table 2.8 and 2.9 show summaries of the components of reproductive isolation that contribute to total isolation for the individual ACO population-crosses. Due to the large block effects with the adult emergence assay, development was not included in this calculation for reproductive isolation. Only two RI-Values were found to be statistically significant out of 55 tests conducted, across all characters and population-crosses.

The ACO_1 populations had a higher focal fecundity than the ACO_5 population with $p = 0.006$. The F_1 hybrid population A_{51} had a greater focal fecundity than its maternal ancestral population ACO_5 with $p = 0.045$. The absolute reproductive isolation value for the $ACO_5 \times ACO_1$ cross is -0.223, which actually is evidence for hybrid vigor, not hybrid underdominance. For all other ACO crosses, the absolute reproductive isolation value is zero (Table 2.10 Part B-section iii).

B Treatment

Productivity: Figures 2.6 and 2.7 shows the productivity results for the B population-crosses. Four population-crosses gave a result that indicated a statistically significant difference in focal productivity between a competitor and an ancestral population (Table 2.12 and 2.13). The F_1 hybrid population B_{32} had a lower fecundity than the ancestral population B_2 (about 13 eggs per female for $\mathbf{F_1}$ B_{54} vs. about 16 eggs per female for B_2), with $p = 0.0001$. Cohabiting B_{54} population had a lower fecundity than the B_4 population (about 8 eggs per female for **c** B_{54} vs. about 12 eggs per female for B_4), with $p = 0.039$. And finally, the $\mathbf{F_1}$ B_{54} had higher fecundity than both ancestral populations B_4 and B_5 (about 16 eggs per female for $\mathbf{F_1}$ B_{54} vs. about 12 eggs per female for B_4 and about 9 eggs per female for B_5), with $p = 0.045$ and $p < 0.0001$, respectively.

Tables 2.11 summarize the mate choice data. These data were analyzed using a chi-square test. Only one mate choice test was found to be statistically significant out of 10 tests conducted. When given a choice between B_1 males and B_5 males, B_5 females preferred to mate with B_5 males ($p = 0.012$). B_5 males were chosen by B_5 females 62 times out of 99 trials compared to B_1 males who were chosen 37 times.

Reproductive isolation values: Table 2.14 provides a summary of reproductive isolation for the CO population-crosses. Out of five pair-wise comparisons, two were found to have some

degree of reproductive isolated and one pair-wise comparison was found to have hybrid vigor. The absolute reproductive isolation value for the $B_2 \times B_3$ cross is 0.075. The absolute reproductive isolation value for the $B_5 \times B_1$ cross is 0.190. The absolute reproductive isolation value for the $B_4 \times B_5$ cross is -0.177. Absolute reproductive isolation value is calculated as zero for the remaining crosses.

CO Treatment

Fecundity and Development: The CO population crosses did not exhibit any statistically significant reproductive isolation effects for focal fecundity (Table 2.16 and 2.17).

Tables 2.15 summarize the mate choice data. Two mate choice tests were found to be statistically significant out of 10 tests conducted. When given a choice between CO_3 males and CO_4 males, CO_3 females preferred to mate with CO_3 males ($p = 0.028$). CO_3 males were chosen by CO_3 females 56 times out of 91 trials compared to CO_4 males who were chosen 35 times.

In addition, when given a choice between CO_1 males and CO_5 males, CO_5 females preferred to mate with CO_5 males ($p = 0.021$). CO_5 males were chosen by CO_5 females 66 times out of 108 trials compared to CO_1 males who were chosen 42 times.

Reproductive isolation values: Table 2.18 provides a summary of reproductive isolation for the CO population-crosses. Two pair-wise comparisons were found to have some degree of reproductive isolated out of five tests conducted. The absolute reproductive isolation value for the $CO_3 \times CO_4$ is 0.190. The absolute reproductive isolation value for the $CO_5 \times CO_1$ is 0.182. Absolute reproductive isolation value is calculated as zero for all other crosses.

Discussion

Overview of the salience of the results

This study involves four major kinds of results: (1) genome-wide sequencing; (2) functional phenotypic differentiation; (3) assessment of general underdominance among crosses; and (4) the success of individuals in scenarios in which they invade the population of their pair-wise crosses. These four major kinds of results are across three different sets of five populations (ACO_{1-5}, B_{1-5}, and CO_{1-5}), each of which have long shared a common selection regime. In the case of the replicate B populations, they have shared a selection regime for more than 30 years, in turn more than 850 generations. In the case of the replicate CO populations, they have shared this particular selection regime for more than 20 years, although their total period of shared evolutionary history is also more than 30 years. Because of their longer generation length, the CO replicate populations have had only about 500 generations of shared evolutionary history. There are no outbred, Mendelian, laboratory populations known to us that are as well-suited to testing the null theory of speciation.

We have tried to give the null theory every reasonable opportunity to demonstrate its merits under laboratory conditions. In the first part of our results, based on genome-wide sequencing of the 15 study populations, we show that there is sufficient genetic differentiation between the ACO and B populations to make them plausible candidates for populations undergoing incipient reproductive isolation. In particular, we emphasize that their F_{ST} values among replicate populations are comparable to those found among *D. melanogaster* populations that have colonized Australia. Given this finding, at a minimum those 10 populations constitute

useful test material for null mechanisms of speciation to generate incipient reproductive isolation, if not the entire system of 15 populations.

In the second part of our results, we find statistically marginal evidence for some functional phenotypic differentiation among replicate populations within selection regimes. We do not wish to overstate the importance of these particular results

In the third part of our results, we find no evidence for general underdominance among crosses of replicate populations within selection regimes. We do have some evidence for directional dominance, benefiting hybrids, among the ACO and CO populations. However, that directional dominance is not on a scale that suggests extensive hybrid vigor, and thus inbreeding depression among ACO and CO populations. As a whole, these results suggest that hundreds of generations of parallel lab evolution have not produced consistent patterns of "epistatic coadaptation" specific to individual replicate populations.

That leaves only the fourth part of our analysis to decide the merits of the null theories, the part which concerned the success of individuals in scenarios in which they invade another population within their evolutionary group. This is an extensive body of data, covering four different kinds of character: developmental speed, mating success, fecundity, and early-adult mortality rates. We find some weak evidence for incipient reproductive isolation in the B and CO population crosses involving crosses B_2-B_3, and B_5-B_1, as well as crosses CO_3-CO_4 and CO_5-CO_1, respectively. Please note that the subscripts for the B populations do not indicate any shared ancestry with the CO populations, so these results are independent from each other. For the ACO crosses we find only negative RI-values. Specifically, we find that hybridization is favored between ACO_1 and ACO_5 crosses. These results are of the same kind as our tests for hybrid vigor or underdominance, in third subsection of the Results, which provided some

evidence for hybrid vigor rather than underdominance. Surveying these results by themselves, they do not supply a striking case for the action of null mechanisms for speciation. But it is important to put these results in context, which we attempt next.

Limitations of the present study

The present study was conducted using unique material: three sets of five-fold replicated populations that have shared evolutionary histories over more than 30 years. Genome-wide sequencing has provided us with clear evidence that ten of these populations have achieved levels of differentiation, within shared evolutionary histories, comparable to that found among some natural populations of *D. melanogaster*. Thus we were at least moderately hopeful that some signal of incipient reproductive isolation might be found under these conditions.

Compared to our study, we find only modestly-powered studies of null mechanisms of speciation in the literature of evolutionary biology. The primary prerequisite for a proper null speciation study is that populations are adapting under uniform selection. It is debatable whether there are ever exactly equivalent shared environments among populations in natural habitats. What may seem to the observer as similar or even identical conditions between populations, may in fact involve profound differences that are not initially observed. What complicates matters further is uncertainty over the consistency of the strength of selection over time. In addition, there is the issue of scale of replication, as it is usually difficult to find numerous independent instances of natural populations adapting even to nominally "similar" environments. Coupled with the potential for variation in the occurrence of population-size bottlenecks, conclusions drawn from studies from natural populations will usually be suggestive at best.

To address the uncontrolled variables posed by conducting experiments in nature, biologists have brought populations into the laboratory environment. In the laboratory environment, two general approaches of studying reproductive isolation are commonly used. The first, and by far the most popular, approach is the intentional inbreeding of natural populations that may be incipient species. Several inbred lines are created of each population and then are subsequently studied both genetically and phenotypically (vid. Coyne and Orr 2003). However, because these studies are done in the laboratory, measures of reproductive isolation are not given an appropriate ecological context (Schluter 2000).

Using data from Coyne and Orr's (1989; 1997) literature survey of inbred strains of *Drosophila* sister species, Schluter (2000) compared the strength of reproductive isolation between pairs of Hawaiian and continental *Drosophila*. Despite the high speciation rate among Hawaiian *Drosophila,* no difference was detected in the average strength of reproductive isolation in crosses between Hawaiian and continental *Drosophila* populations. However, there is some uncertainty with respect to the reliability of this test, as Schluter (2000) points out, because nothing is known about the strength of divergent selection in either Hawaiian or continental *Drosophila*.

The second approach to studying reproductive isolation in the lab has used experimental evolution. Most of these efforts involved subjecting populations of *Drosophil*a or *Musca* to a series of population bottlenecks to study the effect of drift on prezygotic isolation (reviewed in Rice and Hostert 1993). The results of these studies were mixed, with a majority of pairwise combinations showing no prezygotic isolation. The few studies that demonstrated a positive result for prezgotic isolation used populations that were produced by crossing flies collected from different locations (Dodd and Powell 1985; Ringo et al. 1985). It is not clear whether these

reproductive incompatibilities arose via drift or were the product of inherited variants from different locations (cf. Rundle et al. 1998). Overall these types of studies generally suffer from poor replication, with just two or in some cases three replicates per evolutionary treatment. In addition, the majority of these published studies measure either prezygotic or postzygotic barriers, but rarely both (for review see Coyne and Orr 2003; Nosil 2012).

From the preceding discussion, our tests of the null theory of speciation are evidently both more extensive and more stringent than others in the literature on speciation. We find no signal for reproductive isolation among populations that share parallel selection histories over decades in the laboratory, and hundreds of generations of carefully sustained parallel selection with moderate effective population sizes. In context, then, the null theory of speciation does not appear to be particularly plausible, given our findings and their standing relative the literature as a whole.

Alternative experimental strategies for testing null models of speciation

But any single experimental paradigm has its scientific limitations. In particular, our study was carried out after less than 1,000 generations of parallel evolution. What if we were to repeat our study using these same populations in 50 years' time? We would argue that an 80-year *Drosophila* experimental evolution project that featured long-sustained parallel evolution across thousands of generations is extremely unlikely to be germane to the evolution of populations in nature. Most importantly, the likelihood that nature sustains closely parallel selection regimes of isolated populations on such a scale strikes us as remote, particularly from the standpoint of number of generations, which would greatly exceed 1,000. Surely accidents of meteorology and ecology would produce material ecological and demographic differences

among fish populations living in separate streams, or among non-flying terrestrial animals isolated on separate islands, on that evolutionary time scale?

Another experimental design feature that might be varied is effective population size. We use moderately large effective population sizes in our research, at least by the standards of Drosophila experimental evolution (vid. Mueller et al. 2013). Much larger populations will be provided with a greater number of unique mutations after splitting from a common ancestral population. That would tend to foster evolutionary genetic differentiation. On the other hand, much larger populations would undergo much slower rates of genetic drift and would feature more stringent selection for or against new alleles. Therefore, we are not convinced that performing our kind of experiments with much larger effective population sizes would yield better prospects for null mechanisms of speciation.

By contrast, much smaller effective population sizes might generate enough genetic drift, accidental fixation of functionally important allele combinations, and thus ultimately more selection for epistatically coadapted combinations of gene combinations, all of which might make null mechanisms of speciation work better. Fortunately for this scenario, it is a lot easier to sustain numerous relatively inbred lab populations than the larger populations we have studied here. Therefore, this is an experimental strategy that might, with enough evolutionary time, produce the kind of underdominance required to make null mechanisms of speciation work. Note however that, by our standards, previous studies of crosses among inbred lines do not achieve the level of replication or appropriateness to constitute the kind of test we have in mind here.

Conclusion

Our overall conclusion is that we have found no evidence whatsoever for the null theory of speciation. In the absence of ecologically distinct selection regimes, over time periods of less than 1,000 generations, experimental evolution of outbred Mendelian populations does not readily produce incipient reproductive isolation, we suggest. Our experiments are not exhaustive. Nor do they investigate other, potentially promising, experimental paradigms for the production of incipient reproductive isolation in the absence of differences in patterns of selection. But we do claim that this work has provided the strongest tests yet of the validity of null theories of speciation. And further, such theories did not fare well in our tests.

Acknowledgements

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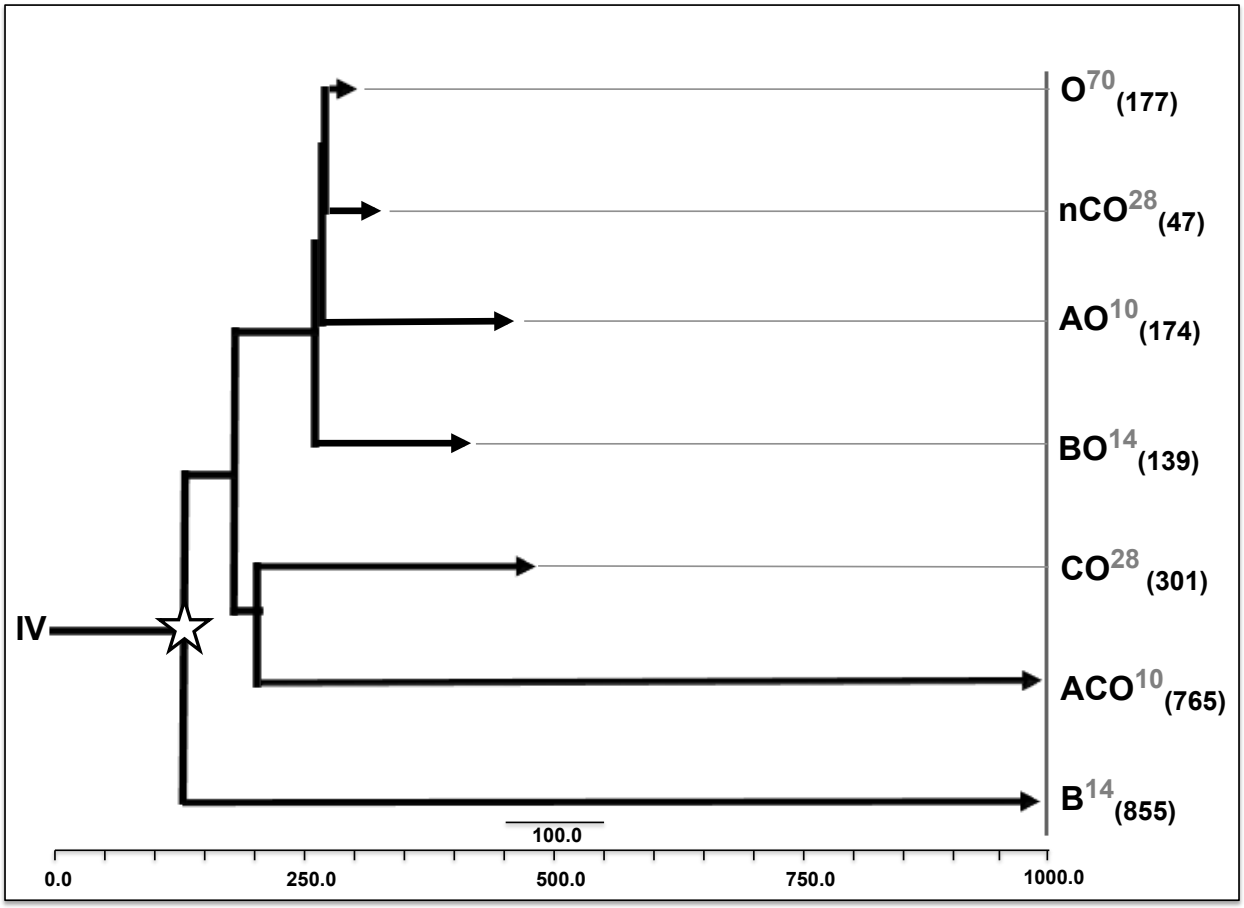

Figure 2.1. Collection of allopatric Drosophila laboratory populations derived from a single outbred population ("IV") in early1980 (star). Each selection regime was imposed on five populations. The X-axis gives the number of generations evolving under laboratory conditions. The Y-axis shifts indicate changes in selection regimes, with the life-cycle length of each selection regime indicated by the superscript, and the number generations evolving under distal selection regime indicated by the subscript.

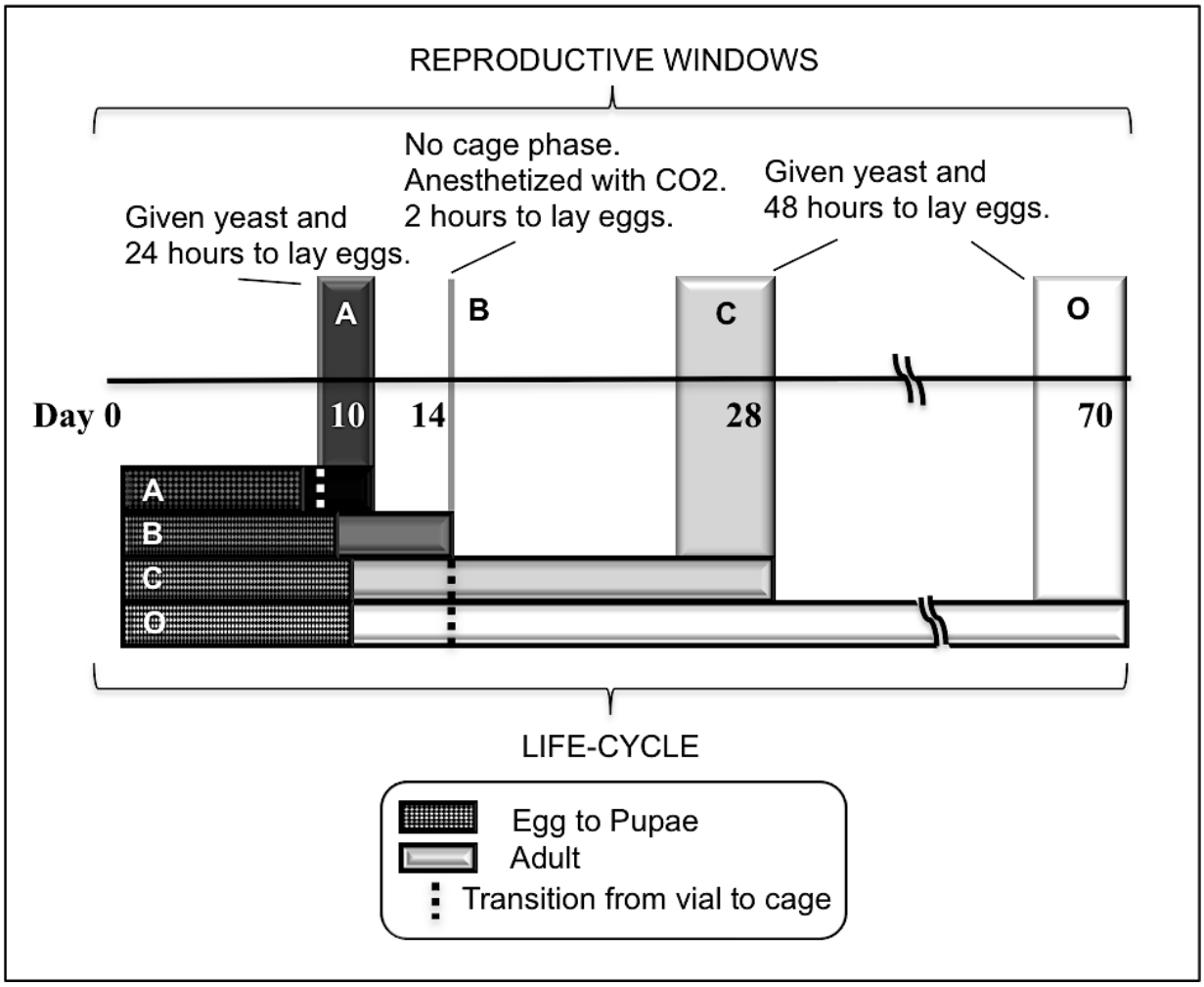

Figure 2.2. Distinct selection regimes imposed on five-fold replicated groups of outbred populations. The primary difference between selection regimes is the time interval (reproductive window) when eggs are collected to establish the next generation. Only A-type, B-type, and C-type populations were used in this study.

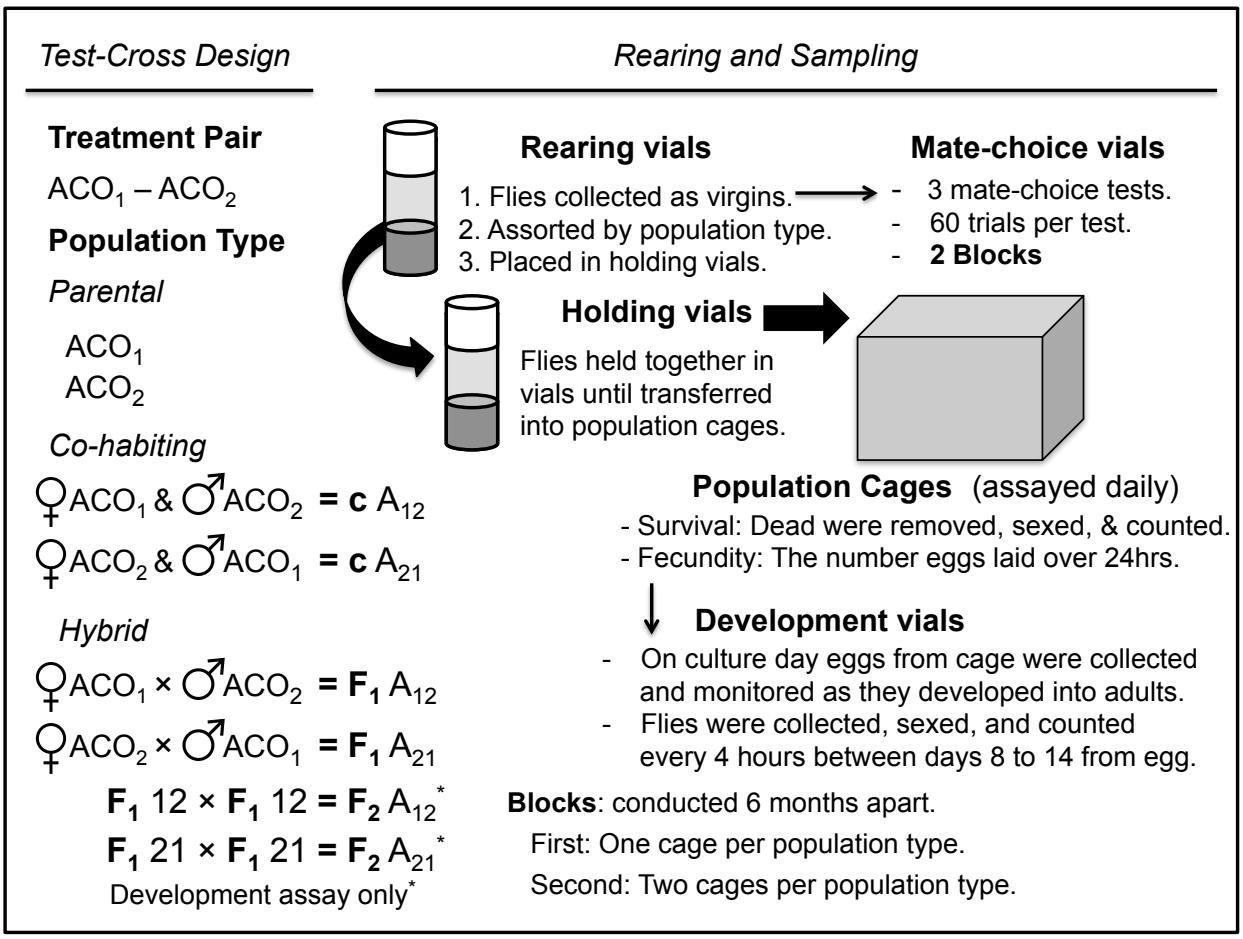

Figure 2.3. Overall experimental structure. There was a systematic experimental design difference between the two blocks. Single assay cohorts were used for each test-cross in block 1, while two assay cohorts were used for block 2.

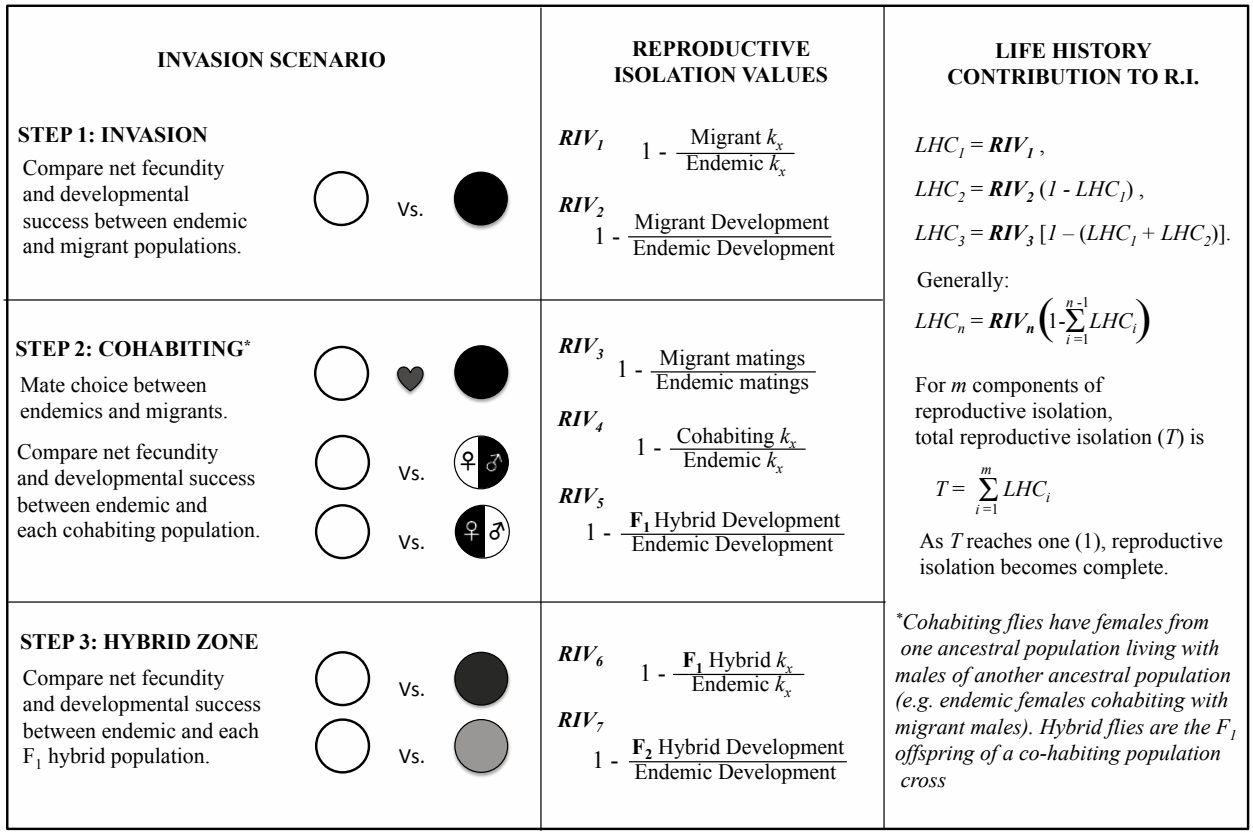

Figure 2.4. Schematic of a secondary contact scenario conceived as a model system for incipient reproductive isolation between long-allopatric populations. A composite measure of reproductive isolation was calculated using sequential life-history components.

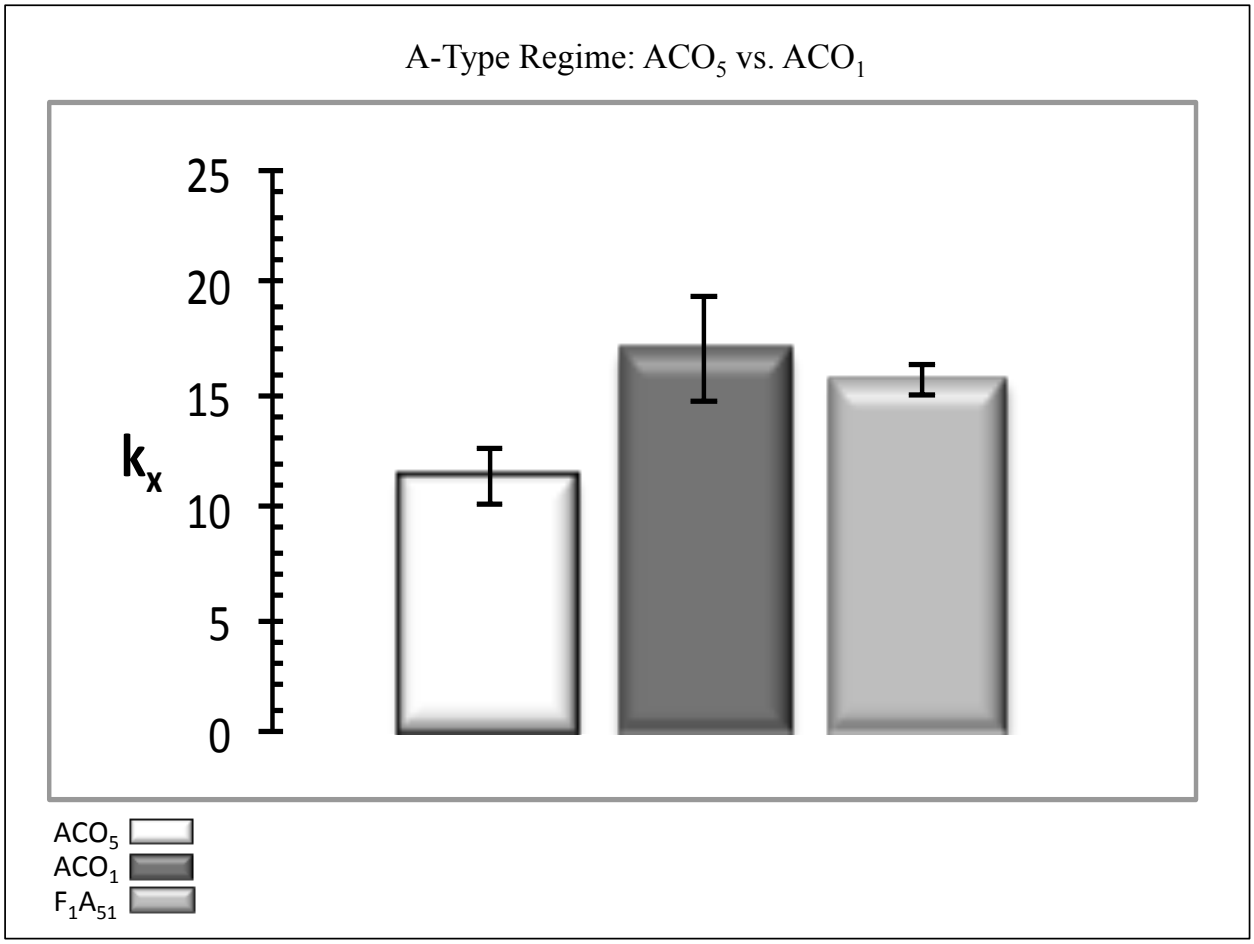

Figure 2.5. Adult age-specific focal fecundity k_x of the populations from the ACO_5-ACO_1 population cross ($k_x = l_x\, m_x$, where l_x is probability of survival to age x and m_x is fecundity at age x). Points represent average fecundity per population in each population cross.

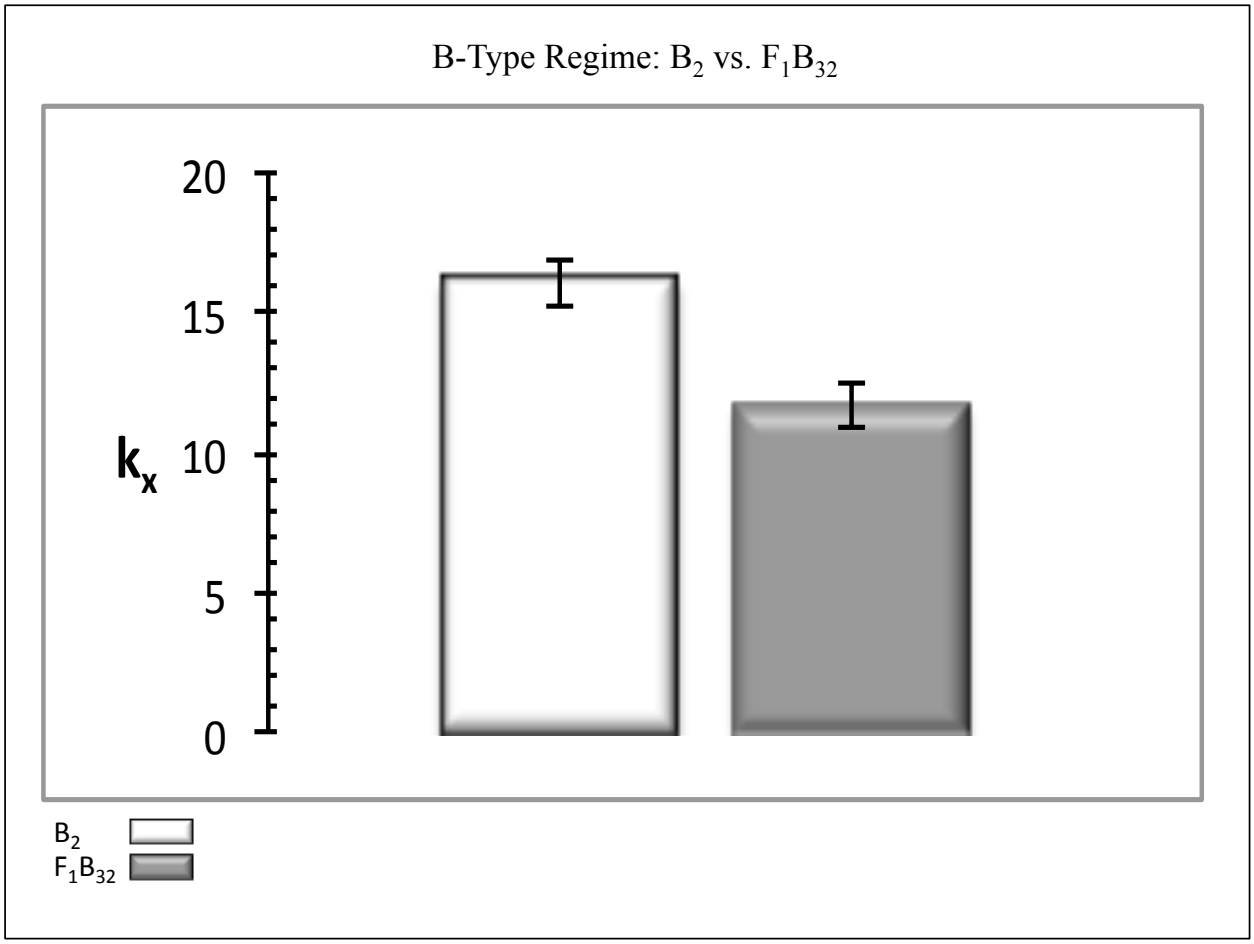

Figure 2.6. Productivity k_x of 2 population types from the B_2-B_3 cohort cross ($k_x = l_x\, m_x$, where l_x is probability of survival to age x and m_x is fecundity at age x). Error bars indicate standard error.

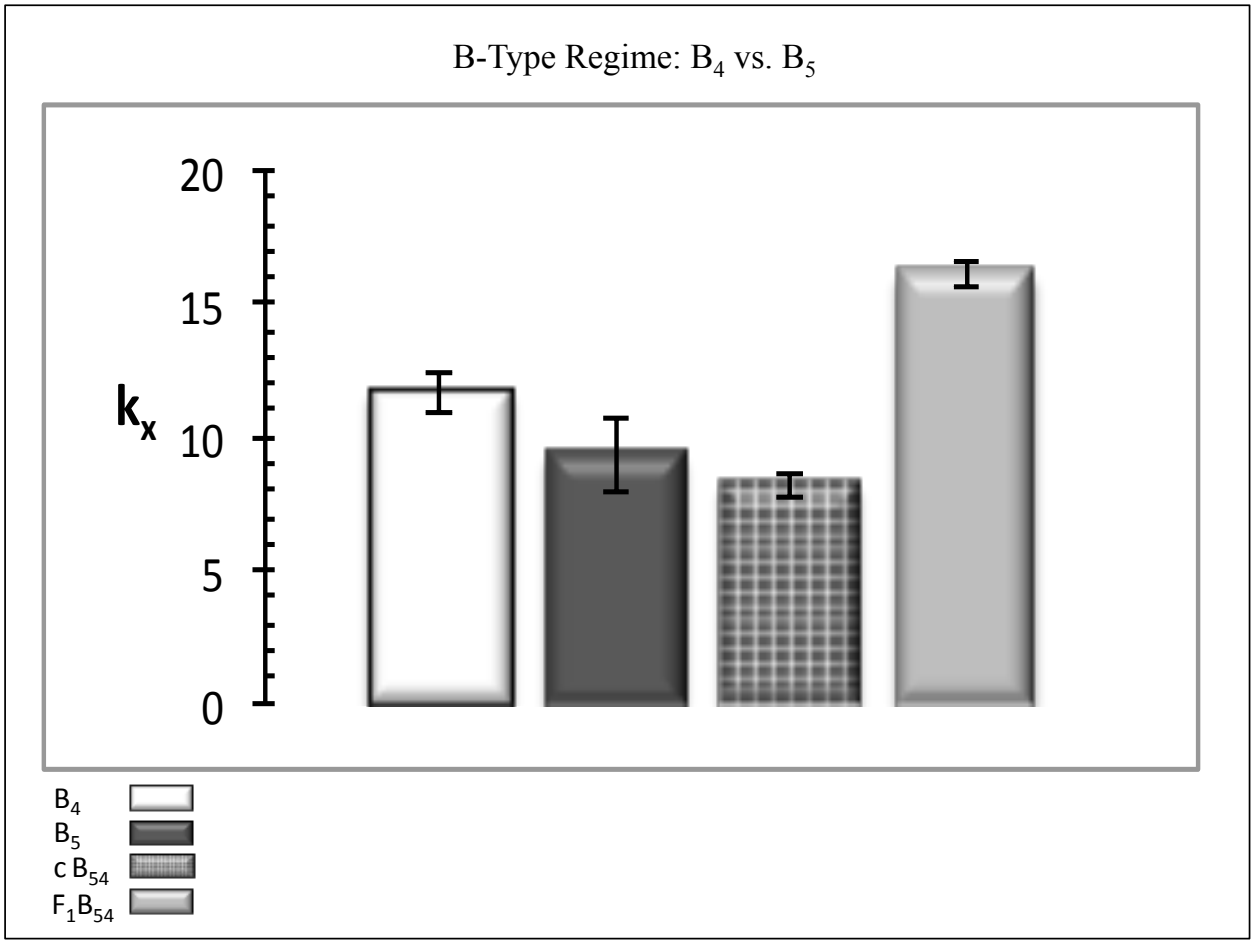

Figure 2.7. Productivity k_x of 2 population types from the B_4-B_5 cohort cross ($k_x = l_x\, m_x$, where l_x is probability of survival to age x and m_x is fecundity at age x). Error bars indicate standard error.

Table 2.1. Mean F_{ST} estimates across the major chromosome arms amongst treatment groups and between cohort pairs based on SNP frequencies. *The number of generations at the time of DNA sampling for the F_{ST} analysis and Block 1 of round-robin population crosses.

Treatment	Mean F_{ST}	Cohort Pair	Mean F_{ST}	Generations*
ACO_{1-5}	0.060	ACO_1 - ACO_2	0.032	822
		ACO_2 - ACO_3	0.043	
		ACO_3 - ACO_4	0.039	
		ACO_4 - ACO_5	0.036	
		ACO_5 - ACO_1	0.035	
B_{1-5}	0.062	B_1 - B_2	0.034	838
		B_2 - B_3	0.035	
		B_3 - B_4	0.038	
		B_4 - B_5	0.039	
		B_5 - B_1	0.041	
CO_{1-5}	0.040	CO_1 - CO_2	0.022	347
		CO_2 - CO_3	0.021	
		CO_3 - CO_4	0.024	
		CO_4 - CO_5	0.030	
		CO_5 - CO_1	0.028	

Table 2.2. Summary of the data analysis of differentiation among ancestral populations for average mortality rate.

Mortality Rate (Ave)								
Structured	Treatment	Gender	Level	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
Part A. 2 Block Analysis	ACO	Male	Population	4	0.0030	0.0007	1.264	NS
			Pop.:Blocks	5	0.0030	0.0006	0.684	0.646
			Residuals	10	0.0086	0.0009		
		Female	Population	4	0.0062	0.0016	2.061	NS
			Pop.:Blocks	5	0.0038	0.0008	0.530	0.749
			Residuals	10	0.0143	0.0014		
	B	Male	Population	4	0.0006	0.0001	2.947	NS
			Pop.:Blocks	5	0.0002	0.0000	1.089	0.423
			Residuals	10	0.0004	0.0000		
		Female	Population	4	0.0007	0.0002	3.971	NS
			Pop.:Blocks	5	0.0002	0.0000	1.068	0.433
			Residuals	10	0.0004	0.0000		
	CO	Male	Population	4	0.0001	0.0000	0.301	NS
			Pop.:Blocks	5	0.0005	0.0001	2.532	0.099
			Residuals	10	0.0004	0.0000		
		Female	Population	4	0.0004	0.0001	0.534	NS
			Pop.:Blocks	5	0.0008	0.0002	0.978	0.476
			Residuals	10	0.0017	0.0002		
Part B. 3 Block Analysis	ACO	Male	Population	4	0.0001	0.0000	0.169	NS
			Pop.:Blocks	5	0.0008	0.0001	2.454	0.057
			Residuals	10	0.0005	0.0000		
		Female	Population	4	0.0001	0.0000	0.224	NS
			Pop.:Blocks	5	0.0012	0.0001	1.059	0.446
			Residuals	10	0.0017	0.0001		
	B	Male	Population	4	0.0010	0.0003	7.684	$p < 0.01$
			Pop.:Blocks	5	0.0003	0.0000	0.595	0.794
			Residuals	10	0.0008	0.0001		
		Female	Population	4	0.0010	0.0002	5.026	NS
			Pop.:Blocks	5	0.0005	0.0000	1.465	0.244
			Residuals	10	0.0005	0.0000		
	CO	Male	Population	4	0.0075	0.0019	4.667	NS
			Pop.:Blocks	5	0.0040	0.0004	0.377	0.938
			Residuals	10	0.0161	0.0011		
		Female	Population	4	0.0156	0.0039	6.812	$p<0.01$
			Pop.:Blocks	5	0.0057	0.0006	0.285	0.975
			Residuals	10	0.0301	0.0020		

Table 2.3. Summary of the data analysis of differentiation among ancestral populations for total fecundity.

Total Fecundity k_x							
Structure	Treatments	Level	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
Part A. 2 Block Analysis	ACO	Population	4	2.393	0.598	1.493	NS
		Pop.:Blocks	5	2.005	0.401	0.679	0.649
		Residuals	10	5.901	0.590		
	B	Population	4	5.526	1.382	6.004	NS
		Pop.:Blocks	5	1.150	0.230	0.417	0.827
		Residuals	10	5.523	0.552		
	CO	Population	4	2.534	0.634	0.641	NS
		Pop.:Blocks	5	4.940	0.988	0.560	0.729
		Residuals	10	17.636	1.764		
Part B. 3 Block Analysis	ACO	Population	4	5.444	1.361	4.303	NS
		Pop.:Blocks	10	3.163	0.316	0.480	0.878
		Residuals	15	9.885	0.659		
	B	Population	4	7.920	1.980	12.328	$p<0.01$
		Pop.:Blocks	10	1.606	0.161	0.332	0.958
		Residuals	15	7.256	0.484		
	CO	Population	4	3.521	0.880	1.660	NS
		Pop.:Blocks	10	5.301	0.530	0.372	0.941
		Residuals	15	21.404	1.427		

Table 2.4. Summary of the data analysis of differentiation among ancestral populations for focal fecundity. Focal productivity k_x was used for the B populations.

Focal Fecundity k_x							
Structure	Treatments	Level	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
Part A. 2 Block Analysis	ACO	Population	4	45.46	11.37	0.5427	NS
		Pop.:Blocks	5	104.75	20.95	4.636	0.0189
		Residuals	10	45.2	4.52		
	B	Population	4	40.58	10.14	0.7953	NS
		Pop.:Blocks	5	63.75	12.75	1.017	0.457
		Residuals	10	125.41	12.54		
	CO	Population	4	72.8	18.19	0.7824	NS
		Pop.:Blocks	5	116.3	23.25	0.419	0.825
		Residuals	10	555.1	55.51		
Part B. 3 Block Analysis	ACO	Population	4	113.1	28.286	1.775	NS
		Pop.:Blocks	10	159.4	15.938	2.182	0.0836
		Residuals	15	109.6	7.306		
	B	Population	4	73.2	18.301	2.181	NS
		Pop.:Blocks	10	83.93	8.393	0.92	0.541
		Residuals	15	136.89	9.126		
	CO	Population	4	89.1	22.27	1.628	NS
		Pop.:Blocks	10	136.8	13.68	0.263	0.981
		Residuals	15	781.1	52.07		

Table 2.5. Summary of the data analysis of potential hybrid underdominance. Focal fecundity is the closest characteristic to fitness. Focal productivity k_x was used for the B populations.

Focal Fecundity k_x									
Confidence Intervals									
	ACO			B			CO		
	Low		High	Low		High	Low		High
	2.30		3.37	-0.472		0.472	1.24		4.01
Population	Block			Block			Block		
	1	2	3	1	2	3	1	2	3
ACO_1	18.10	13.43	17.65	12.88	18.63	15.28	17.01	23.29	22.98
ACO_2	13.79	13.84	14.93	13.92	17.51	17.76	17.85	20.03	21.19
ACO_3	10.79	18.94	20.53	11.70	15.89	16.55	21.86	17.83	18.87
ACO_4	13.75	13.31	11.91	13.48	13.05	11.74	26.66	21.14	22.83
ACO_5	9.27	13.31	12.51	12.05	12.15	13.02	18.13	19.31	19.91
$\mathbf{F_1}$ A_{12}	18.17	14.00	14.24	12.66	22.25	15.02	19.07	31.51	30.06
$\mathbf{F_1}$ A_{21}	22.83	11.40	11.58	13.49	18.86	18.29	25.32	32.02	34.30
$\mathbf{F_1}$ A_{23}	13.82	15.83	17.42	14.36	16.17	17.24	15.52	23.88	22.29
$\mathbf{F_1}$ A_{32}	12.35	18.22	20.90	9.11	14.57	16.35	12.33	22.69	19.62
$\mathbf{F_1}$ A_{34}	12.89	12.67	19.38	14.30	17.21	16.75	21.74	20.14	18.07
$\mathbf{F_1}$ A_{43}	17.77	22.09	19.26	15.70	13.10	12.93	23.02	22.64	23.47
$\mathbf{F_1}$ A_{45}	13.28	16.26	14.98	11.25	13.01	10.55	17.56	21.28	18.81
$\mathbf{F_1}$ A_{54}	12.00	17.38	16.73	6.67	9.86	11.04	23.35	24.71	24.14
$\mathbf{F_1}$ A_{15}	13.48	14.35	13.66	13.09	15.01	15.55	23.12	14.61	17.22
$\mathbf{F_1}$ A_{51}	15.23	15.39	17.07	17.42	14.44	15.01	24.01	14.35	16.29

Table 2.6. Summary of the data analysis of ACO developmental success in adult emergence within nine days. A-type flies develop in vials for nine full days before successfully emergent adults are transferred into population cages to begin laying eggs. The percent of adults that eclosed by the end of nine days are reported below. Populations are labeled **c** for cohabiting, $\mathbf{F_1}$ and $\mathbf{F_2}$ for hybrid types. Asterisks indicate p-values below .05. °Results were consistent between blocks.

ACO Cross	Population Types		Eclosed %		Block 1	Eclosed %		Block 2
	Pop. 1	Pop. 2	Pop. 1	Pop. 2	*p*-value	Pop. 1	Pop. 2	*p*-value
1-2	ACO_1	**c** A_{12}	0.85	0.76	<0.00001*	0.87	0.9	0.015*
	ACO_1	$\mathbf{F_1}$ A_{12}	0.85	0.70	<0.00001*	0.87	0.9	<0.00001*
	ACO_2	**c** A_{21}	0.81	0.81	0.994	0.89	0.93	<0.00001*
	ACO_2	$\mathbf{F_1}$ A_{21}	0.81	0.80	0.672	0.89	0.94	<0.00001*
2-3	ACO_2	**c** A_{23}	0.96	0.96	0.987	0.66	0.80	<0.00001*
	ACO_2	$\mathbf{F_1}$ A_{23}	0.96	0.94	0.237	0.66	0.68	<0.00001*
	ACO_3	**c** A_{32}	0.9	0.81	<0.00001*	0.64	0.77	<0.00001*
	ACO_3	$\mathbf{F_1}$ A_{32}	0.9	0.94	0.011*°	0.64	0.67	0.023*°
3-4	ACO_3	**c** A_{34}	0.64	0.52	<0.00001*	0.6	0.66	<0.00001*
	ACO_3	$\mathbf{F_1}$ A_{34}	0.64	0.57	0.000*	0.6	0.77	<0.00001*
	ACO_4	**c** A_{43}	0.34	0.59	<0.00001*	0.78	0.72	0.000*
	ACO_4	$\mathbf{F_1}$ A_{43}	0.34	0.7	<0.00001*	0.78	0.55	<0.00001*
4-5	ACO_4	**c** A_{45}	0.97	0.92	<0.00001*	0.91	0.92	0.381
	ACO_4	$\mathbf{F_1}$ A_{45}	0.97	0.94	0.002*	0.91	0.94	0.000*
	ACO_5	**c** A_{54}	0.93	0.93	0.866	0.95	0.91	<0.00001*
	ACO_5	$\mathbf{F_1}$ A_{54}	0.93	0.96	0.002*	0.95	0.92	0.000*
5-1	ACO_5	**c** A_{51}	0.93	0.95	0.119	0.85	0.74	<0.00001*
	ACO_5	$\mathbf{F_1}$ A_{51}	0.93	0.91	0.115°	0.85	0.84	0.332°
	ACO_1	**c** A_{15}	0.87	0.98	<0.00001*	0.79	0.8	0.785
	ACO_1	$\mathbf{F_1}$ A_{15}	0.87	0.94	<0.00001*	0.79	0.76	0.006*

Table 2.7. Results of the mate-choice assay for prezygotic reproductive isolation among ACO populations. Asterisks indicate p-values below .05.

ACO	Female	Male 1	Male 2	Total Matings		*p*-value		
				Male 1	Male 2	Block 1	Block 2	Both
1 vs. 2	ACO_1	ACO_1	ACO_2	52	51	0.674	0.781	0.921
	ACO_2	ACO_2	ACO_1	46	59	0.414	0.327	0.205
2 vs. 3	ACO_2	ACO_2	ACO_3	45	48	0.555	0.307	0.756
	ACO_3	ACO_3	ACO_2	47	34	1.000	0.047*	0.232
3 vs. 4	ACO_3	ACO_3	ACO_4	38	48	0.746	0.248	0.281
	ACO_4	ACO_4	ACO_3	34	40	0.262	0.866	0.485
4 vs. 5	ACO_4	ACO_4	ACO_5	35	39	0.873	0.612	0.642
	ACO_5	ACO_5	ACO_4	41	35	0.446	0.862	0.491
5 vs. 1	ACO_5	ACO_5	ACO_1	39	63	0.108	0.076♭	0.017♭
	ACO_1	ACO_1	ACO_5	67	47	0.152	0.225	0.061

Table 2.8. Components of reproductive isolation for ACO_i invading ACO_j populations. Isolation components vary from zero (no barrier) to one (complete isolation). Negative component values indicate life-history stages at which hybridization is favored. Each RI-value is reported as the average between blocks. Asterisks indicate p-values below .05. [b]Effect of male obscured by the effect of color or the interaction of both male and color.

	ACO_i invades ACO_j					
Pop. Cross R.I. Barrier	Pop 1.	R.I.V.	*p*-value	Pop 2.	R.I.V.	*p*-value
$ACO_2 \rightarrow ACO_1$						
Ancestor k_x	ACO_2	0.161	0.287	ACO_1	-	-
Mate-choice	ACO_2 (F)	-0.284	0.205	ACO_1 (F)	0.016	0.921
Cohabiting k_x	c A_{21}	0.204	0.062	c A_{12}	0.002	1.000
F_1 Hybrid k_x	F_1 A_{21}	0.007	0.996	F_1 A_{12}	0.034	0.999
Total						
$ACO_3 \rightarrow ACO_2$						
Ancestor k_x	ACO_3	-0.055	0.833	ACO_2	-	-
Mate-choice	ACO_3 (F)	0.232	0.232	ACO_2 (F)	-0.095	0.756
Cohabiting k_x	c A_{32}	-0.125	0.996	c A_{23}	0.060	0.938
F_1 Hybrid k_x	F_1 A_{32}	-0.141	0.675	F_1 A_{23}	-0.125	0.998
Total						
$ACO_4 \rightarrow ACO_3$						
Ancestor k_x	ACO_4	0.003	0.922	ACO_3	-	-
Mate-choice	ACO_4 (F)	-0.191	0.485	ACO_3 (F)	-0.256	0.281
Cohabiting k_x	c A_{43}	0.066	0.447	c A_{34}	-0.030	0.964
F_1 Hybrid k_x	F_1 A_{43}	-0.312	0.658	F_1 A_{34}	0.022	0.951
Total						
$ACO_5 \rightarrow ACO_4$						
Ancestor k_x	ACO_5	0.000	0.999	ACO_4	-	-
Mate-choice	ACO_5 (F)	0.134	0.491	ACO_4 (F)	-0.120	0.642
Cohabiting k_x	c A_{54}	-0.183	0.768	c A_{45}	-0.072	0.957
F_1 Hybrid k_x	F_1 A_{54}	-0.294	0.294	F_1 A_{45}	-0.269	0.453
Total						
$ACO_1 \rightarrow ACO_5$						
Ancestor k_x	ACO_1	-0.564	0.006*	ACO_5	-	-
Mate-choice	ACO_1 (F)	0.298	0.061	ACO_5 (F)	-0.273	0.017[b]
Cohabiting k_x	c A_{15}	-0.135	0.141	c A_{51}	-0.397	0.930
F_1 Hybrid k_x	F_1 A_{15}	-0.455	0.642	F_1 A_{51}	-0.274	0.045*
Total						

Table 2.9. Components of reproductive isolation for ACO_j invading ACO_i populations. Isolation components vary from zero (no barrier) to one (complete isolation). Negative component values indicate life-history stages at which hybridization is favored. Each RI-value is reported as the average between blocks. Asterisks indicate p-values below .05. [þ]Effect of male obscured by the effect of color or the interaction of both male and color.

ACO_j invades ACO_i						
Pop. Cross R.I. Barrier	Pop 1.	R.I.V.	*p*-value	Pop 2.	R.I.V.	*p*-value
$ACO_1 \rightarrow ACO_2$						
Ancestor k_x	ACO_1	-0.194	0.287	ACO_2	-	-
Mate-choice	ACO_1 (F)	0.016	0.921	ACO_2 (F)	-0.284	0.205
Cohabiting k_x	c A_{12}	-0.192	0.281	c A_{21}	0.053	0.985
F_1 Hybrid k_x	F_1 A_{12}	-0.154	0.502	F_1 A_{21}	-0.178	0.610
Total						
$ACO_2 \rightarrow ACO_3$						
Ancestor k_x	ACO_2	0.042	0.833	ACO_3	-	-
Mate-choice	ACO_2 (F)	-0.095	0.756	ACO_3 (F)	0.232	0.232
Cohabiting k_x	c A_{23}	0.095	0.266	c A_{32}	-0.092	0.981
F_1 Hybrid k_x	F_1 A_{23}	-0.093	0.969	F_1 A_{32}	-0.094	1.000
Total						
$ACO_3 \rightarrow ACO_4$						
Ancestor k_x	ACO_3	-0.049	0.922	ACO_4	-	-
Mate-choice	ACO_3 (F)	-0.256	0.281	ACO_4 (F)	-0.191	0.485
Cohabiting k_x	c A_{34}	-0.030	1.000	c A_{43}	0.081	0.960
F_1 Hybrid k_x	F_1 A_{34}	0.007	1.000	F_1 A_{43}	-0.323	0.125
Total						
$ACO_4 \rightarrow ACO_5$						
Ancestor k_x	ACO_4	-0.129	0.999	ACO_5	-	-
Mate-choice	ACO_4 (F)	-0.120	0.642	ACO_5 (F)	0.134	0.491
Cohabiting k_x	c A_{45}	-0.044	0.996	c A_{54}	-0.224	0.923
F_1 Hybrid k_x	F_1 A_{45}	-0.317	0.681	F_1 A_{54}	-0.301	0.505
Total						
$ACO_5 \rightarrow ACO_1$						
Ancestor k_x	ACO_5	0.349	0.006*	ACO_1	-	-
Mate-choice	ACO_5 (F)	-0.273	0.017[þ]	ACO_1 (F)	0.298	0.061
Cohabiting k_x	c A_{51}	0.104	0.106	c A_{15}	0.262	0.889
F_1 Hybrid k_x	F_1 A_{51}	0.184	0.988	F_1 A_{15}	0.066	0.347
Total						

Table 2.10. Summary of reproductive isolation for test-crosses under the A-type culture-regime environments. Isolation components vary from zero (no barrier) to one (complete isolation). Negative component values indicate life-history stages at which hybridization is favored. Part A lists life history contribution to reproductive isolation calculated with RI-values regardless of statistical significance. Part B lists life history contribution to reproductive isolation calculated with only RI-values that are statistically significant.

ACO_i invades ACO_j

Invasion	R.I. Barrier	Round Robin Crosses				
		2 → 1	3 → 2	4 → 3	5 → 4	1 → 5
A)	Ancestor k_x	0.161	-0.055	0.003	0.000	-0.564
i. All R.I. Values	Mate-choice	-0.111	0.071	-0.233	-0.017	-0.007
ACO_i→ACO_j	Cohabiting k_x	0.110	-0.017	-0.058	-0.274	-0.537
	F_1 Hybrid k_x	0.007	-0.109	-0.317	-0.656	-0.803
	Total	0.167	-0.109	-0.605	-0.947	-1.912
		1 → 2	2 → 3	3 → 4	4 → 5	5 → 1
	Ancestor k_x	-0.194	0.042	-0.049	-0.129	0.349
ii. All R.I. Values	Mate-choice	-0.163	0.067	-0.223	0.035	0.020
ACO_j→ACO_i	Cohabiting k_x	-0.074	-0.015	0.032	-0.134	0.100
	F_1 Hybrid k_x	-0.249	-0.118	-0.183	-0.402	0.077
	Total	-0.680	-0.024	-0.423	-0.630	0.545
iii. All R.I.V. Between Populations		-0.256	-0.066	-0.514	-0.789	-0.684
		2 → 1	3 → 2	4 → 3	5 → 4	1 → 5
B)	Ancestor k_x	0.000	0.000	0.000	0.000	-0.564
i. Stat. Sig. R.I.V.	Mate-choice	0.000	0.000	0.000	0.000	0.000
ACO_i→ACO_j	Cohabiting k_x	0.000	0.000	0.000	0.000	0.000
	F_1 Hybrid k_x	0.000	0.000	0.000	0.000	-0.231
	Total	0.000	0.000	0.000	0.000	-0.795
		1 → 2	2 → 3	3 → 4	4 → 5	5 → 1
	Ancestor k_x	0.000	0.000	0.000	0.000	0.349
ii. Stat. Sig. R.I.V.	Mate-choice	0.000	0.000	0.000	0.000	0.000
ACO_j→ACO_i	Cohabiting k_x	0.000	0.000	0.000	0.000	0.000
	F_1 Hybrid k_x	0.000	0.000	0.000	0.000	0.000
	Total	0.000	0.000	0.000	0.000	0.349
iii. Stat. Sig. R.I.V. Between Populations		0.000	0.000	0.000	0.000	-0.223

Table 2.11. Results of the mate-choice assay for prezygotic reproductive isolation among B populations. Asterisks indicate p-values below .05.

B	Female	Male 1	Male 2	Total Matings		*p*-value		
				Male 1	Male 2	Block 1	Block 2	Both
1 vs. 2	B_1	B_1	B_2	45	53	0.662	0.484	0.419
	B_2	B_2	B_1	50	51	0.327	0.258	0.921
2 vs. 3	B_2	B_2	B_3	53	49	0.285	0.555	0.692
	B_3	B_3	B_2	53	47	0.345	0.881	0.549
3 vs. 4	B_3	B_3	B_4	57	39	0.058	0.537	0.066
	B_4	B_4	B_3	48	37	0.647	0.217	0.232
4 vs. 5	B_4	B_4	B_5	56	63	0.606	0.696	0.521
	B_5	B_5	B_4	53	52	0.896	0.768	0.922
5 vs. 1	B_5	B_5	B_1	62	37	0.009^*	0.379	0.012^*
	B_1	B_1	B_5	43	59	0.782	0.011^*	0.113

Table 2.12. Components of reproductive isolation for B_i invades B_j populations. Isolation components vary from zero (no barrier) to one (complete isolation). Negative component values indicate life-history stages at which hybridization is favored. Each RI-value is reported as the average between blocks. Asterisks indicate p-values below 0.05. [b]Effect of male obscured by the effect of color or the interaction of both male and color.

	B_i invades B_j					
Pop. Cross R.I. Barrier	Pop 1.	R.I.V.	*p*-value	Pop 2.	R.I.V.	*p*-value
$B_2 \rightarrow B_1$						
Ancestor k_x	B_2	-0.031	1.000	B_1	-	-
Mate-choice	B_2 (F)	-0.070	0.921	B_1 (F)	-0.177	0.419
Cohabiting k_x	c B_{21}	0.081	0.998	c B_{12}	-0.092	0.987
F_1 Hybrid k_x	F_1 B_{21}	0.166	0.907	F_1 B_{12}	0.085	0.999
Total						
$B_3 \rightarrow B_2$						
Ancestor k_x	B_3	0.220	0.123	B_2	-	-
Mate-choice	B_3 (F)	0.090	0.549	B_2 (F)	0.030	0.692
Cohabiting k_x	c B_{32}	0.161	0.132	c B_{23}	0.043	0.983
F_1 Hybrid k_x	F_1 B_{32}	0.299	0.0001*	F_1 B_{23}	0.134	0.375
Total						
$B_4 \rightarrow B_3$						
Ancestor k_x	B_4	0.068	0.431	B_3	-	-
Mate-choice	B_4 (F)	0.225	0.232	B_3 (F)	0.293	0.066
Cohabiting k_x	c B_{43}	0.016	0.998	c B_{34}	-0.043	1.000
F_1 Hybrid k_x	F_1 B_{43}	0.007	0.967	F_1 B_{34}	-0.035	1.000
Total						
$B_5 \rightarrow B_4$						
Ancestor k_x	B_5	0.260	0.326	B_4	-	-
Mate-choice	B_5 (F)	0.024	0.922	B_4 (F)	-0.125	0.521
Cohabiting k_x	c B_{54}	0.283	0.039*	c B_{45}	0.041	0.927
F_1 Hybrid k_x	F_1 B_{54}	-0.281	0.045*	F_1 B_{45}	-0.079	0.984
Total						
$B_1 \rightarrow B_5$						
Ancestor k_x	B_1	0.105	0.983	B_5	-	-
Mate-choice	B_1 (F)	-0.525	0.113	B_5 (F)	0.379	0.012*
Cohabiting k_x	c B_{15}	0.160	0.997	c B_{51}	0.032	0.472
F_1 Hybrid k_x	F_1 B_{15}	0.105	1.000	F_1 B_{51}	-0.008	0.788
Total						

Table 2.13. Components of reproductive isolation for B_j invades B_i populations. Isolation components vary from zero (no barrier) to one (complete isolation). Negative component values indicate life-history stages at which hybridization is favored. Each RI-value is reported as the average between blocks. Asterisks indicate p-values below 0.05. [b]Effect of male obscured by the effect of color or the interaction of both male and color.

	B_j invades B_i					
Pop. Cross R.I. Barrier	Pop 1.	R.I.V.	*p*-value	Pop 2.	R.I.V.	*p*-value
$B_1 \rightarrow B_2$						
Ancestor k_x	B_1	0.029	1.000	B_2	-	-
Mate-choice	B_1 (F)	-0.177	0.419	B_2 (F)	-0.070	0.921
Cohabiting k_x	c B_{12}	-0.058	0.996	c B_{21}	0.105	0.992
F_1 Hybrid k_x	F_1 B_{12}	0.108	0.996	F_1 B_{21}	0.186	0.846
Total						
$B_2 \rightarrow B_3$						
Ancestor k_x	B_2	-0.328	0.123	B_3	-	-
Mate-choice	B_2 (F)	0.030	0.692	B_3 (F)	0.090	0.549
Cohabiting k_x	c B_{23}	-0.271	0.456	c B_{32}	-0.114	1.000
F_1 Hybrid k_x	F_1 B_{23}	-0.146	0.994	F_1 B_{32}	0.069	0.278
Total						
$B_3 \rightarrow B_4$						
Ancestor k_x	B_3	-0.108	0.431	B_4	-	-
Mate-choice	B_3 (F)	0.293	0.066	B_4 (F)	0.225	0.232
Cohabiting k_x	c B_{34}	-0.146	0.243	c B_{43}	-0.082	0.726
F_1 Hybrid k_x	F_1 B_{34}	-0.125	0.510	F_1 B_{43}	-0.075	0.900
Total						
$B_4 \rightarrow B_5$						
Ancestor k_x	B_4	-0.407	0.326	B_5	-	-
Mate-choice	B_4 (F)	-0.125	0.521	B_5 (F)	0.024	0.922
Cohabiting k_x	c B_{45}	-0.396	0.899	c B_{54}	-0.024	0.943
F_1 Hybrid k_x	F_1 B_{45}	-0.525	0.074	F_1 B_{54}	-0.791	<0.0001*
Total						
$B_5 \rightarrow B_1$						
Ancestor k_x	B_5	-0.147	0.983	B_1	-	-
Mate-choice	B_5 (F)	0.379	0.012*	B_1 (F)	-0.525	0.113
Cohabiting k_x	c B_{51}	-0.122	0.882	c B_{15}	0.024	1.000
F_1 Hybrid k_x	F_1 B_{51}	-0.182	0.991	F_1 B_{15}	-0.044	0.998
Total						

Table 2.14. Summary of reproductive isolation for test-crosses under the B-type culture-regime environments. Isolation components vary from zero (no barrier) to one (complete isolation). Negative component values indicate life-history stages at which hybridization is favored. Part A lists life history contribution to reproductive isolation calculated with RI-values regardless of statistical significance. Part B lists life history contribution to reproductive isolation calculated with only RI-values that are statistically significant.

B➔B: Life History Contribution to Reproductive Isolation

Invasion	R.I. Barrier	Round Robin Crosses				
		2 ➔ 1	3 ➔ 2	4 ➔ 3	5 ➔ 4	1 ➔ 5
A)	Ancestor k_x	-0.031	0.220	0.068	0.260	0.105
i. All R.I. Values	Mate-choice	-0.121	0.021	0.243	-0.032	-0.119
B_i➔B_j	Cohabiting k_x	-0.023	0.076	-0.012	0.133	0.158
	F_1 Hybrid k_x	0.122	0.139	-0.027	-0.102	0.096
	Total	-0.053	0.456	0.272	0.259	0.241
		1 ➔ 2	2 ➔ 3	3 ➔ 4	4 ➔ 5	5 ➔ 1
	Ancestor k_x	0.029	-0.328	-0.108	-0.407	-0.147
ii. All R.I. Values	Mate-choice	-0.125	0.124	0.285	-0.081	-0.015
B_j➔B_i	Cohabiting k_x	0.005	-0.233	-0.118	-0.465	0.033
	F_1 Hybrid k_x	0.120	-0.115	-0.118	-1.554	-0.118
	Total	0.029	-0.552	-0.059	-2.507	-0.247
iii. All R.I.V. Between Populations		-0.012	-0.048	0.107	-1.124	-0.003
		2 ➔ 1	3 ➔ 2	4 ➔ 3	5 ➔ 4	1 ➔ 5
B)	Ancestor k_x	0.000	0.000	0.000	0.000	0.000
i. Stat. Sig. R.I.V.	Mate-choice	0.000	0.000	0.000	0.000	0.190
B_i➔B_j	Cohabiting k_x	0.000	0.000	0.000	0.141	0.000
	F_1 Hybrid k_x	0.000	0.150	0.000	-0.100	0.000
	Total	0.000	0.150	0.000	0.042	0.190
		1 ➔ 2	2 ➔ 3	3 ➔ 4	4 ➔ 5	5 ➔ 1
	Ancestor k_x	0.000	0.000	0.000	0.000	0.000
ii. Stat. Sig. R.I.V.	Mate-choice	0.000	0.000	0.000	0.000	0.190
B_j➔B_i	Cohabiting k_x	0.000	0.000	0.000	0.000	0.000
	F_1 Hybrid k_x	0.000	0.000	0.000	-0.396	0.000
	Total	0.000	0.000	0.000	-0.396	0.190
iii. Stat. Sig. R.I.V. Between Populations		0.000	0.075	0.000	-0.177	0.190

Table 2.15. Components of reproductive isolation for CO_i invades CO_j populations. Isolation components vary from zero (no barrier) to one (complete isolation). Negative component values indicate life-history stages at which hybridization is favored. Each RI-value is reported as the average between blocks. Asterisks indicate p-values below 0.05. [b]Effect of male obscured by the effect of color or the interaction of both male and color.

CO_i invades CO_j						
Pop. Cross R.I. Barrier	Pop 1.	R.I.V.	*p*-value	Pop 2.	R.I.V.	*p*-value
$CO_2 \rightarrow CO_1$						
Ancestor k_x	CO_2	0.080	0.819	CO_1	-	-
Mate-choice	CO_2 (F)	-0.052	0.750	CO_1 (F)	0.065	0.632
Cohabiting k_x	c C_{21}	0.086	0.999	c C_{12}	0.011	0.984
F_1 Hybrid k_x	F_1 C_{21}	-0.108	0.956	F_1 C_{12}	0.091	0.972
Total						
$CO_3 \rightarrow CO_2$						
Ancestor k_x	CO_3	-0.429	0.446	CO_2	-	-
Mate-choice	CO_3 (F)	0.080	0.695	CO_2 (F)	-0.106	0.626
Cohabiting k_x	c C_{32}	-0.353	0.420	c C_{23}	-0.167	0.948
F_1 Hybrid k_x	F_1 C_{32}	-0.191	0.818	F_1 C_{23}	-0.371	0.394
Total						
$CO_4 \rightarrow CO_3$						
Ancestor k_x	CO_4	-0.150	0.460	CO_3	-	-
Mate-choice	CO_4 (F)	0.191	0.042 [b]	CO_3 (F)	0.380	0.028*
Cohabiting k_x	c C_{43}	-0.242	0.078	c C_{34}	0.082	0.913
F_1 Hybrid k_x	F_1 C_{43}	-0.220	0.146	F_1 C_{34}	-0.081	0.941
Total						
$CO_5 \rightarrow CO_4$						
Ancestor k_x	CO_5	0.032	1.000	CO_4	-	-
Mate-choice	CO_5 (F)	-0.027	1.000	CO_4 (F)	-0.005	1.000
Cohabiting k_x	c C_{54}	0.067	0.999	c C_{45}	0.052	1.000
F_1 Hybrid k_x	F_1 C_{54}	0.060	1.000	F_1 C_{45}	0.254	0.276
Total						
$CO_1 \rightarrow CO_5$						
Ancestor k_x	CO_1	-0.174	0.823	CO_5	-	-
Mate-choice	CO_1 (F)	-0.132	0.626	CO_5 (F)	0.364	0.021*
Cohabiting k_x	c C_{15}	-0.109	0.998	c C_{51}	-0.065	0.997
F_1 Hybrid k_x	F_1 C_{15}	-0.476	0.250	F_1 C_{51}	-0.469	0.284
Total						

Table 2.16. Components of reproductive isolation for CO_j invades CO_i populations. Isolation components vary from zero (no barrier) to one (complete isolation). Negative component values indicate life-history stages at which hybridization is favored. Each RI-value is reported as the average between blocks. Asterisks indicate p-values below 0.05. [b]Effect of male obscured by the effect of color or the interaction of both male and color.

CO_j invades CO_i						
Pop. Cross R.I. Barrier	Pop 1.	R.I.V.	*p*-value	Pop 2.	R.I.V.	*p*-value
$CO_1 \rightarrow CO_2$						
Ancestor k_x	CO_1	-0.089	0.819	CO_2	-	-
Mate-choice	CO_1 (F)	0.065	0.632	CO_2 (F)	-0.052	0.750
Cohabiting k_x	c C_{12}	-0.073	0.994	c C_{21}	0.000	0.948
F_1 Hybrid k_x	F_1 C_{12}	0.008	0.997	F_1 C_{21}	-0.203	0.289
Total						
$CO_2 \rightarrow CO_3$						
Ancestor k_x	CO_2	0.299	0.446	CO_3	-	-
Mate-choice	CO_2 (F)	-0.106	0.626	CO_3 (F)	0.080	0.695
Cohabiting k_x	c C_{23}	0.187	0.937	c C_{32}	0.062	1.000
F_1 Hybrid k_x	F_1 C_{23}	0.049	1.000	F_1 C_{32}	0.177	0.992
Total						
$CO_3 \rightarrow CO_4$						
Ancestor k_x	CO_3	0.109	0.460	CO_4	-	-
Mate-choice	CO_3 (F)	0.380	0.028*	CO_4 (F)	0.191	0.042 [b]
Cohabiting k_x	c C_{34}	0.166	0.053	c C_{43}	-0.082	0.952
F_1 Hybrid k_x	F_1 C_{34}	0.046	0.950	F_1 C_{43}	-0.066	0.990
Total						
$CO_4 \rightarrow CO_5$						
Ancestor k_x	CO_4	-0.107	1.000	CO_5	-	-
Mate-choice	CO_4 (F)	-0.005	1.000	CO_5 (F)	-0.027	1.000
Cohabiting k_x	c C_{45}	-0.004	1.000	c C_{54}	0.019	0.999
F_1 Hybrid k_x	F_1 C_{45}	0.231	0.276	F_1 C_{54}	0.018	1.000
Total						
$CO_5 \rightarrow CO_1$						
Ancestor k_x	CO_5	0.144	0.823	CO_1	-	-
Mate-choice	CO_5 (F)	0.364	0.021*	CO_1 (F)	-0.132	0.626
Cohabiting k_x	c C_{51}	0.090	0.976	c C_{15}	0.046	0.973
F_1 Hybrid k_x	F_1 C_{51}	-0.275	0.952	F_1 C_{15}	-0.284	0.934
Total						

Table 2.17. Results of the mate-choice assay for prezygotic reproductive isolation among CO populations. Asterisks indicate p-values below .05. [b]Effect of male obscured by the effect of color or the interaction of both male and color.

CO	Female	Male 1	Male 2	Total Matings		*p*-value		
				Male 1	Male 2	Block 1	Block 2	Both
1 vs. 2	CO_1	CO_1	CO_2	57	52	0.285	0.680	0.632
	CO_2	CO_2	CO_1	43	46	0.492	0.739	0.750
2 vs. 3	CO_2	CO_2	CO_3	50	55	0.414	0.889	0.626
	CO_3	CO_3	CO_2	54	50	1.000	0.555	0.695
3 vs. 4	CO_3	CO_3	CO_4	56	35	0.327	0.027*	0.028*
	CO_4	CO_4	CO_3	64	43	0.080[b]	0.267	0.042[b]
4 vs. 5	CO_4	CO_4	CO_5	58	58	0.796	0.789	1.000
	CO_5	CO_5	CO_4	51	51	0.475	0.492	1.000
5 vs. 1	CO_5	CO_5	CO_1	66	42	0.789	0.317	0.021*
	CO_1	CO_1	CO_5	50	55	0.091	0.116	0.626

Table 2.18. Summary of reproductive isolation for test-crosses under the C-type culture-regime environments. Isolation components vary from zero (no barrier) to one (complete isolation). Negative component values indicate life-history stages at which hybridization is favored. Part A lists life history contribution to reproductive isolation calculated with RI-values regardless of statistical significance. Part B lists life history contribution to reproductive isolation calculated with only RI-values that are statistically significant.

CO➔CO: Life History Contribution to Reproductive Isolation

Invasion	R.I. Barrier	Round Robin Crosses				
		2 ➔ 1	3 ➔ 2	4 ➔ 3	5 ➔ 4	1 ➔ 5
A)	Ancestor k_x	0.080	-0.429	-0.150	0.032	-0.174
i. All R.I. Values	Mate-choice	0.007	-0.012	0.324	0.001	0.128
CO_i➔CO_j	Cohabiting k_x	0.063	-0.335	-0.124	0.027	-0.085
	F_1 Hybrid k_x	-0.013	-0.623	-0.211	0.083	-0.479
	Total	0.137	-1.399	-0.161	0.143	-0.611
		1 ➔ 2	2 ➔ 3	3 ➔ 4	4 ➔ 5	5 ➔ 1
	Ancestor k_x	-0.089	0.299	0.109	-0.107	0.144
ii. All R.I. Values	Mate-choice	0.007	-0.012	0.258	-0.036	0.105
CO_j➔CO_i	Cohabiting k_x	-0.021	0.110	-0.005	-0.018	0.053
	F_1 Hybrid k_x	-0.118	0.058	-0.025	0.130	-0.213
	Total	-0.221	0.455	0.337	-0.032	0.089
iii. All R.I.V. Between Populations		-0.042	-0.472	0.088	0.055	-0.261
		2 ➔ 1	3 ➔ 2	4 ➔ 3	5 ➔ 4	1 ➔ 5
B)	Ancestor k_x	0.000	0.000	0.000	0.000	0.000
i. Stat. Sig. R.I.V.	Mate-choice	0.000	0.000	0.190	0.000	0.182
CO_i➔CO_j	Cohabiting k_x	0.000	0.000	0.000	0.000	0.000
	F_1 Hybrid k_x	0.000	0.000	0.000	0.000	0.000
	Total	0.000	0.000	0.190	0.000	0.182
		1 ➔ 2	2 ➔ 3	3 ➔ 4	4 ➔ 5	5 ➔ 1
	Ancestor k_x	0.000	0.000	0.000	0.000	0.000
ii. Stat. Sig. R.I.V.	Mate-choice	0.000	0.000	0.190	0.000	0.182
CO_j➔CO_i	Cohabiting k_x	0.000	0.000	0.000	0.000	0.000
	F_1 Hybrid k_x	0.000	0.000	0.000	0.000	0.000
	Total	0.000	0.000	0.190	0.000	0.182
iii. Stat. Sig. R.I.V. Between Populations		0.000	0.000	0.190	0.000	0.182

CHAPTER 3

Testing the Ecological Speciation Theory

Abstract

The ecological speciation hypothesis suggests that reproductive isolation evolves as result of divergent adaptation in response to natural selection arising from differences between environments. We tested this explanation using two *Drosophila melanogaster* treatments that share a recent common ancestor with five replicate populations per treatment. The two treatments differ chiefly with respect to timing of their reproductive window (egg culture day), with one reproducing early (14-days old) and the other later (28-days old). These five pairs of populations have 204 generations of divergence between them. Between-treatment population crosses were created and analyzed phenotypically using four assays: 1) mate-choice, 2) mortality, 3) fecundity, and 4) development. The data analysis was based on an invasion scenario for the resumption of contact between flies from different lab populations. Migrant and hybrid populations were found to have less fitness when compared to endemic populations under endemic conditions. Overall, we found strong evidence for the role of ecologically divergent selection among allopatric laboratory populations in producing incipient reproductive isolation. The over-arching implication of this work is that this model system has great potential to resolve the underpinnings of speciation.

Introduction

There is some consensus among evolutionary biologists that the primary driving force of speciation is natural selection, as opposed to the "null" scenarios of Chapter Two. The way this theoretical consensus is formulated now is couched in terms of "ecological speciation" hypotheses. First proposed implicitly by Charles Darwin (1859), ecological speciation theory is based on the conjecture that reproductive isolation is caused by divergent phenotypic adaptation in response to differences between environments while populations are separated allopatrically. This line of thinking suggests that reproductive isolation should be more likely to evolve between allopatric populations that have significantly adapted to different environments, compared to allopatric populations that are well adapted to similar environments.

Evidence for the ecological speciation hypothesis has chiefly come from studies employing a "top-down" approach. This approach can be summarized as research that obtains the following types of information: (i) traits under divergent selection among populations living in different environments, (ii) traits that produce some degree of reproductive isolation between these populations, and (iii) evidence for a genetic basis for the differentiation of both of these types of traits (Schluter 2009). Acquiring the third kind of information is the most challenging, but is needed in order to establish that it is selection that has led to reproductive isolation, rather some feature of the different environments that produces phenotypic differentiation without any genetic basis.

Ecological speciation has been inferred from instances of assortative mating involving body size and coloration in fish (McKinnon et al. 2004), beak size in birds (Podos 2001), pollinator preferences (Ramsey et al. 2003) and variation in flowering time (Lowry et al. 2008b).

Ecological speciation has also been inferred from instances of unfit hybrids arising from both disrupted mimicry in butterfly hybrids (Jiggins et al. 2001; Naisbit et al. 2001) and intermediate migration patterns in bird hybrids (Helbig 1991).

Previous studies that have employed divergent parallel selection in replicate populations of *D. melanogaster* have found evidence for reproductive isolation in as little as 18 generations (Robertson 1966a,b; Boake et al. 2003). The signal detected by these studies is primarily prezygotic reproductive isolation. One study tested populations of *Drosophila melanogaster* that had adapted to an environment containing the chemical DDT (Boake et al. 2003). Selection for DDT resistance had been maintained on one line for about 25 years and then relaxed for more than 15 years, though it still retained some DDT resistance at the time of testing. In total these lines had been maintained in allopatry for 600 generations. The selected line had lower egg production and a shorter lifespan than the control line, and both lines had homotypic mate preference. Robertson (1966 a,b) detected postzygotic isolation in allopatric populations adapting to food with and without EDTA for 20 generations, with two replicate populations for each selection regime.

The ecological theory of speciation implies that the number of generations of divergence is not the key parameter in the evolution of reproductive isolation. Instead, with this hypothesis the magnitude of phenotypic differentiation between functional characters provides is the key determinant of reproductive incompatibility.

In order to test the ecological speciation theory, what is needed is a research system that has multiple replicated populations that have undergone contrasting selection regimes that have in turn led to the evolution of significant, functional, phenotypic divergence. In addition, there should be evidence that at least some of these populations are in the initial stages of evolving

reproductive isolation of some type(s). It would be particularly advantageous if such an extensively differentiated evolutionary system had few complications arising from historical accidents of differentiation that might make comparisons of evolving populations confounded by such accidents. For example, if all these populations were derived from a common ancestral population, many historically accidental evolutionary confounds could be precluded.

In our laboratory, we have such a research system. Our stock system is comprised of dozens of populations of *Drosophila melanogaster* divided among distinct selection treatments, with five or six replicate populations maintained for each selection regime (Rose et al., 2004). The estimated effective population size for each of these populations is about 1000 (Mueller et al., 2013). These replicated treatment-groups constitute lineages that have been sustained for as much as 900 generations. Complementing the extensive phenotypic differentiation within our research system is earlier evidence of incipient reproductive isolation among replicate populations from the B treatment (Long et al., 2006). Long et al. (2006) performed reciprocal crosses between the six IV and B populations, which had been maintained under identical conditions for 637 generations at the time of their experiments. They found seven of the 30 crosses with 'foreign' mates resulted in significant reductions in female components of fitness, whereas two resulted in significant increases in female components of fitness, compared to local matings.

For the 10 populations that we are particularly concerned with in this Chapter, the two selection treatments differ chiefly with respect to the length of their discrete generations: that is, the life-cycle from the egg-laying that starts one generation to the egg-laying that starts the next generation (see Figure 3.1). Here, we present data on incipient reproductive isolation between the ten populations that have recent common ancestors, split into two groups of five each that

have recently been subjected to contrasting selection regimes: BO and nCO. Between-treatment crosses were subjected to four assays to test the strength of both prezygotic and postzygotic reproductive barriers: (1) mate-choice, (2) mortality, (3) fecundity, and (4) development rate.

Materials and Methods

Experimental Populations

Experimental evolutionary history: This study uses outbred lab populations of *Drosophila melanogaster* selected for different patterns of age-specific reproduction. All the flies used in this study ultimately originate from an ancestral "IV" population first collected from South Amherst, MA in 1975 by Phillip Ives (vid. Rose 1984), and then cultured in the lab using two-week discrete generations. These ancestral IV flies were subsequently used in February 1980 to create five "O" (old) replicate lines (Rose 1984). The IV flies were also used to found five additional "B" (baseline) populations in February, 1980, populations which have since been cultured using the same protocol as the IV line from which they were derived (see Figure 3.2).

Culture regimes: Over subsequent years, additional lineages were derived from the O populations using three distinct culture regimes, of which two are studied here: "B" and "C" (see Figure 3.1). ***B*** *culture regime*: the five BO populations spend 14 days in 8-dram vials, and are then allowed 1-2 hours in fresh vials to oviposit before adults are discarded. ***C*** *culture regime:* the five nCO populations develop in vials for 14 days prior to being transferred to Plexiglass cages. C flies are then given 48 hours to oviposit before eggs are collected on day 28. All populations are supplied with food made from cooked bananas, barley malt, yeast, corn syrup,

and agar. The populations that spend time in cages are also supplied with live yeast on the medium surface prior to egg laying.

Test-Cross Experimental Design

Overall experimental structure: Two sets of between-treatment crosses were performed using the five BO and five nCO populations, one under BO (B-type) and one under nCO (C-type) culture-regime conditions. The two sets of crosses were repeated twice, with blocks approximately 6 months apart, which mitigated the impact of random environmental and handling effects on the results. There was a systematic experimental design difference between the two blocks. Single assay cohorts were used for each test-cross in block 1, while two assay cohorts were used for block 2. See Figure 3.3 for an overview.

Matched-subscript crossing system: Crosses between populations from different selection regimes were performed in a "matched-subscript" fashion: $BO_1\times nCO_1$, $BO_2\times nCO_2$, $BO_3\times nCO_3$, $BO_4\times nCO_4$, and $BO_5\times nCO_5$. [Note that when BO and nCO populations share a subscript, they are recently derived from an O population that was coded with the same subscript; thus the subscript pairing is not an incidental feature of this experimental design. However, the derivations of BO and nCO populations were initiated about nine O generations apart in evolutionary time.] For each such cross, three types of flies were assayed: ancestor ("**a**"), cohabiting ("**c**"), and first-generation hybrid ("$\mathbf{F_1}$") flies. A second-generation hybrid ("$\mathbf{F_2}$") was also used, but only for the development duration rate assay. Ancestor flies were obtained from crosses between males and females from a single ancestral population (e.g. all flies sampled from BO_1, in the case of the cross between BO_1 and nCO_1 flies.). Cohabiting flies have females from one ancestral population living with males of another ancestral population (e.g. BO_1 females

cohabiting with nCO_1 males). First-generation hybrid flies are the F_1 offspring of a co-habiting population cross (e.g. all F_1 $BO_1 \times nCO_1$ flies are true hybrids between populations BO_1 and nCO_1).

Rearing and sampling of assay cohorts: D. melanogaster cultures were initiated (day 0) in 25×95 mm vials containing 20 ml of banana/agar/yeast media at a density of 70 eggs per natal vial for each population test-cross.

In order to maintain culture-regime-specific conditions throughout each crossing assay, special natal vials were created. These natal vials were made of two components: a 23×25mm cap and a 25×95 tube. The cap containing fly medium was inserted into the tube to create a vial of standard dimensions. After reaching the appropriate development stage, larvae would then climb the walls of the tube to pupate.

Virgin adults were collected using light CO_2 anesthesia as they eclosed from their pupal cases on day 9 for BO populations, and day 10 for nCO populations. Flies were sexed, crossed, and then placed into holding vials that consisted of a cap from the natal vials inserted into a clean tube. Population test-crosses were made by combining 25 virgin flies of each gender in the females' natal-capped holding vial, ten vials per population-cross, with 500 flies total for each such cross.

Adults were allowed to mate and freely interact in the females' natal vials until the normal culture day: day 14 for the assays conducted in the B-type environment. In the C-type environment assays, flies were transferred into cages at day 14, and then eggs were collected from those cages on days 26 to 28, from egg. These experimental procedures closely mimicked the culture regime experienced by the ancestral populations (see Figure. 3.1). Thus the assays

performed in this study provide a reasonable estimate of fitness under the culture conditions that each type of fly had experienced for hundreds of generations.

Overview of our analytical strategy

The analysis of our data is based on a specific scenario for the resumption of contact between flies from different lab populations, conceived as a model system for incipient reproductive isolation between long-allopatric populations. As this scenario is somewhat complicated, we have provided both a schematic, shown in Figure 3.4, and the following verbal summary.

If two populations have long been divided by a major geographic barrier, the likelihood that a single migrant will undermine their reproductive isolation is remote. What we have in mind instead is the following scenario.

Step One. A propagule from one geographical area migrates to the other geographical area, where this propagule consists of enough individuals so that it does not suffer from notable inbreeding depression. Note, however, that this initial migration is not assumed to immediately lead to hybridization. Thus the initial analytical question is how well this propagule group can survive under the selective conditions imposed in the geographical area to which it has newly migrated.

Step Two. Over some part of its range in its new geographical area, the migrant propagule group cohabits with the endemic population that has long undergone adaptation to that geographical area. During this phase of the process, matings may occur between endemic and propagule individuals specifically in the zone of cohabitation. At this point, *prezygotic* components of reproductive isolation come into play.

Step Three. As a result of hybridization events, some part of the geographical area inhabited by migrant and endemic individuals constitutes a hybrid zone, in which hybrids and individuals with uncrossed parental genomes constitute a mixed population. At this point, the relative fitness of hybrid progeny compared to individuals with uncrossed parental genomes plays an important evolutionary role. This relative fitness difference can then be assayed with respect to *postzygotic* components of reproductive isolation.

Reproductive Isolation Assays

Our assays tested for both prezygotic and postzygotic reproductive isolation, comparing hybrids to uncrossed individuals for each assay. The prezygotic characters tested were (i) survival to the time of mating and (ii) mate choice. The postzygotic characters tested were (i) fecundity and (ii) development. Uncrossed and hybrid individuals are tested simultaneously by deriving the hybrids from the previous parental generation.

Life-history assays covered the entire range of ages during which any of the tested populations are maintained in our lab, from day 0 (from egg) to day 28 (from egg). Selection-regime "focal" fitness was calculated from data collected specifically during the reproductive window of each treatment's generation cycle.

Mate choice assay: Two mate-choice tests were used: (1) a female from an endemic population was given the choice to mate with one of two suitors: a male from her own population and a male from a migrant population; and (2) same procedure as test (1), but with a migrant female as the choosing female instead of an endemic female. Each male was given colored yeast paste to ingest for identification purposes, with rotating combinations of colors among types of male. The flies were given two hours in which to mate. A successful mating event was scored

when a male mounted a female for thirty seconds or more. Mate choice assays were conducted at 24 hours from eclosion using virgin flies. If females did not mate at all within the two hours, the experimental trial was discarded. Males were classified as (i) mated or not mated, (ii) marked or not marked. Sixty choice-assays were performed for each type of test, per block.

Adult survival assay: Flies from holding vials were transferred to population cages at both treatments' normal day of transfer out of their rearing vials: day 14. Population cages were surveyed for dead flies before food plates were replaced each day. The dead flies were removed, then sexed, and their number recorded. Each cage was initiated with 500 flies.

Net fecundity assay: Each assay cage contained a single Petri dish of food medium. Almost all of the eggs were laid in or on that Petri dish. During the adult survival assay, the Petri dish was removed from the cage daily. The removed Petri dish was rinsed with bleach solution in order to collect all the laid eggs onto a membrane placed within a Buchner funnel. The membrane was then placed on a flatbed scanner and photographed. The number of eggs laid was counted from this photographic image using ImageJ software. Net fecundity at a particular age is normally rendered as k_x ($k_x = l_x\, m_x$, where l_x is probability of survival to age x and m_x is fecundity at age x).

Development assay: In the cross assay conducted using the nCO culture regime, 20 vials of ~70 eggs were collected from population cages on the normal culture day 28. For nCO treatments a fraction of the number eggs that laid during focal fecundity were used for development. For the BO population crosses, all eggs that were laid in ten vials during a two-hour period were used for the development assay. The number of eggs that developed into adults was recorded every 4 hours from day 8 to day 14, the last day on which adults are collected from vials. The number of adults that eclosed during the development duration assay was added to the

focal fecundity total. For BO populations, development duration and focal fecundity are not mutually exclusive and thus the number of adults that emerge by day 14 is considered focal productivity and reported as k_x.

Reproductive Isolation Values

We quantified reproductive isolation between populations using a composite measure derived from the test-cross data. Our component measures of Reproductive Isolation Value (RIV) specify the strength of reproductive isolation inferred from each test-cross assay. This component index of reproductive isolation was calculated using the method proposed by Coyne and Orr (1989; 1997)

$$RIV_n = 1 - \frac{competitor}{maternal\,ancestor} \qquad (1)$$

where the subscript *n* refers to the specific character under study (e.g. mate choice). All these component indices of isolation reflect statistically significant differences between "competitor" (e.g. hybrid) and ancestral individuals in each test cross. RIV estimates are expected to vary between negative and positive values, where one is complete reproductive isolation. Scenarios in which hybridization is favored, as a consequence of disassortative mating or hybrid vigor, result in negative reproductive isolation values.

To calculate a composite measure of reproductive isolation, we used the method proposed by Ramsey et al. (2003): where the "life-history contribution" ("*LHC"*) of a component of reproductive isolation value (*RIV*) at stage *n* in the life history is calculated in the following manner:

$$LHC_1 = RIV_1, \qquad (2)$$

$$LHC_2 = RIV_2\left(1 - LHC_1\right), \text{ and} \qquad (3)$$

$$LHC_3 = RIV_3\left[1 - (LHC_1 + LHC_2)\right]. \qquad (4)$$

Generally: $$LHC_n = RIV_n\left(1 - \sum_{i=1}^{n-1} LHC_i\right). \qquad (5)$$

In this parameterization, a particular component reproductive barrier is taken to eliminate gene flow that has not already been prevented by previous components of reproductive isolation. To calculate total reproductive isolation in our study, four sequential life-history stage components were used: 1) focal fecundity of the ancestor populations; 2) mate choice between ancestor-population flies; 3) focal fecundity of the cohabiting flies; and 4) focal fecundity of the F_1 hybrids. For *m* components of reproductive isolation, total reproductive isolation (*T*) is

$$T = \sum_{i=1}^{m} LHC_i . \qquad (6)$$

As *T* reaches one (1), reproductive isolation becomes complete.

Data Analysis

Mate-choice data: The counts of males in each of the possible cells from this experiment were analyzed using a chi-square test. Marking was necessary to distinguish between males from different population crosses. However, since it may impact the females' preference, we controlled for this by rotating colors amongst types of males. Therefore, we were able to test whether mating status is independent of marking status in each experiment.

Survival and Fecundity data: We tested for differences in survival and fecundity due to population-cross status over relative and absolute ages using a linear mixed effects model. The observations consisted of fecundity at a particular age (*t*) but within a small age interval. These age intervals were chosen to span the ages such that all comparison populations still had live

flies. Within each interval, survival or fecundity rates were modeled by a straight line and allowing population type (j= 1 (**a** 1), , 2 (**a** 2), , 3 (**c** 1), 4 (**c** 2), 5 ($\mathbf{F_1}$ 1), 6 ($\mathbf{F_1}$ 2)) to affect the intercept of that line but not the slope. However, slopes were allowed to vary between intervals. As with the other analyses, cages (i=1,…,12) were assumed to contribute random variation to these measures. With this notation, the survival (or fecundity) at age-t, interval-k, selection regime-j and population-i, is y_{ijkt} and is described by,

$$y_{ijkt} = \alpha + \beta_k + \delta_j\gamma_j + (\omega + \pi_k\delta_k)t + \delta_k\delta_j\mu_{jk} + c_i + \varepsilon_{ijkt} \qquad (7)$$

where δ_s =0 if s=1 and 1 otherwise and c_i, and ε_{ijkt} are independent standard normal random variables with variance σ_c^2 and σ_ε^2 respectively. The effects of selection on the intercept are assessed by considering the magnitude and variance of both γ_j and μ_{jk}.

Development data: Successful adult emergence prior to culture day was analyzed with the same analysis as fecundity using a linear mixed effects model. The observations consisted of developmental success percentage at a particular age (t) but within a small time interval. These time intervals were chosen to span the development window prior to culture day. Within each interval, developmental success percentage were modeled by a straight line and allowing population type (j= 1 (**a** 1), , 2 (**a** 2), , 3 (**c** 1), 4 (**c** 2), 5 ($\mathbf{F_1}$ 1), 6 ($\mathbf{F_1}$ 2)) to affect the intercept of that line but not the slope. However, slopes were allowed to vary between intervals. As with the other analyses, population cages (i=1,…,12) were assumed to contribute random variation to these measures. With this notation, the developmental success at age-t, interval-k, selection regime-j and population-i, is y_{ijkt} and is described by,

$$y_{ijkt} = \alpha + \beta_k + \delta_j\gamma_j + (\omega + \pi_k\delta_k)t + \delta_k\delta_j\mu_{jk} + c_i + \varepsilon_{ijkt} \qquad (7)$$

where δ_s =0 if s=1 and 1 otherwise and c_i, and ε_{ijkt} are independent standard normal random variables with variance σ_c^2 and σ_ε^2 respectively. The effects of selection on the intercept are assessed by considering the magnitude and variance of both γ_j and μ_{jk}.

Results

It should be noted here that development wasn't incorporated in the invasion scenario. Under B-type regime conditions, development duration and focal fecundity are not mutually exclusive and thus the number of adults that emerge by day 14 is considered focal productivity and reported as k_x. For populations tested under C-type regime conditions no statistical differences in development were found (p =1.000).

In addition, reported in this Results section are the life-history contribution (LHC) to reproductive isolation for each assay, averaged across all five replicates. LHC calculations are presented in two ways: (1) with all RI-values regardless of statistical significance; and (2) with only statistically significant RI-values. LHC averages using all RI-values, method (1), are shown in order to present all the "noise" obscuring the state of potential incipient reproductive isolation across every assay. The normal practice in the field (e.g. Ramsey et al. 2003) is to take non-significant signals of reproductive isolation as zero, method (2). Interestingly there are only minor differences between the ultimately calculated levels for overall RI obtained using the two methods. For that reason, it is not of much scientific significance that the RI values calculated using method (2) were used to draw general conclusions.

Step One: Initial Success of Uncrossed Migrant Propagule Groups

Figure 3.5 shows the fecundity results of the net reproductive success of nCO populations under B conditions and Figure 3.6 presents the net reproductive success of B populations under C conditions. In each case, the net reproductive success of the "endemic" ancestor is presented alongside that of the supposed migrant ancestor. These data were analyzed using a linear mixed effects model with observations of k_x nested in blocks. A Tukey test was used to correct for multiple comparisons.

The key statistical finding is that, for every population comparison, the endemic ancestor outperformed the migrant ancestor in the number of eggs laid per female. In the B-type regime, the BO populations laid on average about 13 eggs per female while the migrant nCO populations laid on average about 6.3 eggs per female. In each case the difference in net reproductive success between endemic and migrant populations was statistically significant ($p^{1\times1} = .03$, $p^{2\times2} < .0001$, $p^{3\times3} = .0004$, $p^{4\times4} < .0001$, $p^{5\times5} < .0001$). In the C-type regime, the nCO populations laid on average about 23.2 eggs per female, compared to migrant BO populations who laid on average about 11 eggs per female. In each case, the difference in net reproductive success between endemic and migrant populations was statistically significant ($p^{1\times1} = .03$, $p^{2\times2} < .0001$, $p^{3\times3} = .0004$, $p^{4\times4} < .0001$, $p^{5\times5} < .0001$).

Table 3.1 presents the components reproduction isolation values for the net reproductive success of nCO populations under B conditions and Table 3.2 presents the components reproduction isolation values for net reproductive success of B populations under C conditions. For fecundity, the reproductive isolation value (RIV) was calculated as:

$$RIV_1 = 1 - \frac{Migrant\ ancestor\ fecundity\ (kx)}{Endemic\ ancestor\ fecundity\ (kx)}$$

Table 3.3 part B presents the life history contribution (LHC) to reproduction isolation for the net reproductive success of nCO populations under B conditions and Table 3.4 part B presents the life history contribution to reproduction isolation for net reproductive success of B populations under C conditions. For the estimate of premating reproductive isolation between the BO and nCO populations we use the average life history contribution RI-value across all population comparisons. RI-values estimates are expected to vary between zero and one, where one is complete reproductive isolation. The average premating RI-value under the B-type regime is 0.506 ± .06. The average premating RI-value under the C-type regime is 0.514 ± .07. Overall, we find a clear disadvantage facing nCO individuals subjected to B culture conditions, as well as a clear disadvantage facing BO individuals subjected to C culture conditions. These results indicate that, in terms of our scenario, propagule groups of each type will face difficulty maintain themselves under their new environmental conditions.

Step Two: Effects of Cohabitation and Hybridization on Net fecundity

The next step in our scenario features the formation of zones in which hybridization might take place. There are two characters that we have assayed which matter at this point: mate choice preferences and net reproductive success of between-type matings.

Table 3.5 summarizes the mate choice data under the two conditions of cohabitation. These data were analyzed using a chi-square test. Only one mate choice test was found to be statistically significant out of 10 tests conducted. When given a choice between BO_1 males and nCO_1 males, BO_1 females preferred to mate with BO_1 males (p =.022). BO_1 males were chosen by BO_1 females 67 times out of 110 trials compared to nCO_1 males who were chosen 43 times. For mate-choice, the reproductive isolation value was calculated as:

$$RIV_2 = 1 - \frac{frequency\ of\ heterospecific\ matings}{frequency\ of\ homospecific\ matings}$$

This estimate of prezygotic isolation between BO and nCO populations in B-type regime conditions is 0.021 ± .02 (see Table 3.3 part B). This estimate of prezygotic isolation between BO and nCO populations in C-type regime conditions is 0.011 ± .01 (see Table 3.4 part B).

Figure 3.7 shows the fecundity results of the net reproductive success of cohabiting populations under B conditions and Figure 3.8 shows the net reproductive success of cohabitating populations under C conditions. Cohabiting flies have females from one ancestral population living with males of another ancestral population, labeled **c** nB (nCO females with BO males) and **c** Bn (BO females with nCO males). In each case, the net reproductive success of the "endemic" ancestor is presented alongside that of both cohabiting populations. These data were analyzed using a linear mixed effects model with observations of k_x nested in blocks. A Tukey test was used to correct for multiple comparisons.

The key statistical findings were that, for every population comparison, the endemic stock outperformed the cohabiting individuals when the mated females were of migrant origin. In the B-type environment, the BO populations on average laid 13 eggs per female compared to 7 eggs laid per female by the **c** nB populations. In each case the difference in net reproductive success between BO and **c** nB populations was statistically significant ($p^{1\times1} = .026$, $p^{2\times2} < .0001$, $p^{3\times3} = .028$, $p^{4\times4} = .0005$, $p^{5\times5} < .0001$). In addition, in the B-type environment, the BO_4 population laid more eggs than the reciprocal cohabiting population **c** Bn_4, 11.4 eggs per female to 7.8 eggs per female ($p = .008$). In the C-type environment, the nCO populations laid on average 23.1 eggs per female compared to the **c** Bn populations output of 8.4 eggs per female. In each case, the difference in net reproductive success between nCO and **c** Bn populations was statistically significant (for all 5 replicate population comparisons, $p = <.0001$).

Table 3.1 presents the components of reproductive isolation for the net reproductive success of cohabiting populations under B conditions and Table 3.2 presents the components of reproductive isolation for net reproductive success of cohabiting populations under C conditions. For fecundity, the reproductive isolation value (RIV) was calculated as:

$$RIV_3 = 1 - \frac{Cohabiting\ pop.fecundity\ (kx)}{Endemic\ ancestor\ fecundity\ (kx)}$$

For the estimate of postzygotic reproductive isolation between the endemic and cohabiting populations, we use the average life history contribution RI-Value across all population comparisons. The average postzygotic RI-Value between cohabiting and endemic populations under the B-type regime is 0.086 ± .01. The average postzygotic RI-Value between cohabiting and endemic populations under the C-type regime is 0.139 ± .02.

Overall, these results reveal a general absence of mate preferences, with one exception. In addition, the data analysis suggests that it is chiefly the maternal genotype that determines the productivity of any particular mating.

Step Three. Relative Fitness of Hybrids

Given the production of hybrid genotypes in zones which potentially permit hybridization, we can ask how these hybrids fare relative to the individuals who come from the populations that have long adapted to the selective regime of that nominal "geographical area."

Figures 3.9 and 3.10 shows the data for the performance of hybrid genotypes relative to individuals who come from the populations that have long adapted to the selective regime of that nominal "geographical area." These data were analyzed using a linear mixed effects model with observations of k_x nested in blocks. A Tukey test was used to correct for multiple comparisons.

The key findings were that only a few individual comparisons between the endemic ancestral populations and F_1 hybrids were shown to be statistically significant. The F_1 hybrids are the offspring of a cohabiting population cross, with **c** Bn producing $\mathbf{F_1}$ Bn and **c** nB producing $\mathbf{F_1}$ nB. In the B-type environment the BO_4 population on average laid 11.4 eggs per female compared to 8.3 eggs laid per female by the $\mathbf{F_1}$ nB population ($p = .037$). The BO_4 population also outperformed the $\mathbf{F_1}$ Bn hybrid who laid only 7.3 eggs per female ($p = .001$). Population BO_5 also outperformed its hybrid $\mathbf{F_1}$ nB by laying 11.2 eggs per female compared to 7.2 eggs per female ($p < .0001$). In the C-type environment the nCO_3 population laid on average 25.9 eggs per female compared to the $\mathbf{F_1}$ Bn population with 17.6 eggs per female ($p < .0001$) and $\mathbf{F_1}$ nB population with 13.6 eggs per female ($p = .008$).

Table 3.1 presents the components reproductive isolation for the net reproductive success of hybrid populations under B conditions and Table 3.2 presents the components reproductive isolation for net reproductive success of hybrid populations under C conditions. For fecundity, the reproductive isolation value (RIV) was calculated as:

$$RIV_4 = 1 - \frac{F1\ hybrid\ pop.fecundity\ (kx)}{Endemic\ ancestor\ fecundity\ (kx)}$$

For the estimate of postzygotic reproductive isolation between the endemic and hybrid populations, we use the average life history contribution RI-Value across all population comparisons. The average postzygotic RI-Value between cohabiting and endemic populations under the B-type environment is 0.028 ± .02. The average postzygotic RI-Value between hybrid and endemic populations under the C-type environment is 0.015 ± .02.

Reproductive isolation among specific population crosses within selection regimes

In Table 3.3 part B and 3.4 part B is a summary of the life history contribution values for all crosses tested in the B-type and C-type environments. For populations tested in the B-type regime, overall reproductive isolation value averaged across all BO and nCO population comparisons is 0.642 ± .07. The overall reproductive isolation value averaged across all BO and nCO populations tested in the C-type environment is 0.679 ± .07. Overall reproductive isolation between replicate populations tested between environments is 0.661 ± .03 (See Table 3.6 part B).

Discussion

Overview of the salience of the results

The results of this study are relatively clear. Among the five independent pairs of nCO and BO populations, we have clear evidence for the evolution of environment-dependent, incipient, reproductive isolation. That is to say, nCO groups do not do as well under B conditions and produce hybrids that are inferior, relative to individuals from BO populations. Quantitatively, this effect is indicated by an aggregate reproductive isolation value ("RIV") of .642 ± .07. Likewise, BO groups do not do as well as under C conditions and produce hybrids that are inferior, relative to individuals from nCO populations. Quantitatively, this effect is indicated by an aggregate reproductive isolation value ("RIV") of .679 ± .07.

Several things are notable about this result. Firstly, these five pairs of populations have a total arc of evolutionary divergence that is only 204 generations, a relatively brief period in evolutionary time. In terms of calendar years under tropical conditions, this might be as little six to ten years. This is a fairly rapid evolution of incipient reproductive isolation. Secondly, these

five pairs of populations have known levels of genome-wide differentiation for SNPs. Specifically, their pairwise F_{ST} values are: $0.038^{1\times1}$, $0.040^{2\times2}$, $0.040^{3\times3}$, $0.038^{4\times4}$, and $0.038^{5\times5}$.

When the strong signal of reproductive isolation found here is compared to the weak signals of reproductive isolation found in Chapter Two ecological speciation appears to be a demonstrably more potent evolutionary mechanism for the production of incipient reproductive isolation. In addition to this greater potency, the relative speed with which divergent ecological selection has brought about this level of reproductive isolation can be compared to the much longer period over which null mechanisms of speciation were given the opportunity to act in the case of the ACO and B populations of Chapter Two. Chronologically, those ACO and B populations were separated from their last common ancestors decades earlier. In evolutionary time, their total branch-length distances are on the order of ten times greater than those separating the five pairs of nCO_i and BO_i populations, specifically 1698 and 1712 generations versus 204 generations. [These last numbers refer to the number of generations separating these populations at the time of the last experimental block for the experiments of both Chapters 2 and 3. In addition, these numbers are very close to the generation numbers at which samples were taken for whole-genome sequencing, the genomic data from the F_{ST} were calculated.]

At face value, then, the results of this Chapter 3 appear to support ecological speciation as the more plausible speciation mechanism among Mendelian populations. The degree to which such a strong conclusion needs to be qualified is the concern of the next subsection of this Discussion.

Limitations of the present study

Naturally enough, any experimental evolution study of a topic as broad as speciation is fraught with limitations and concerns. We will now supply a provisional list of such concerns, together with some discussion of how germane they are.

While the overall study of this doctoral thesis features a wide range of populations with respect to patterns of evolutionary differentiation, the present Chapter studies just five replicate cases of incipient reproductive isolation involving a total of ten populations. While this might be criticized as very limited replication, even by the standards of Chapter Two, we would claim that the tests of this Chapter Three feature high-quality replication. That is to say, unlike the other studies of ecological speciation known to us (e.g. Schluter 2001, 2009; Via 2009), we have very closely parallel replicate populations. These populations share (i) the same common overall ancestor, the Ives population, (ii) comparable levels of genome-wide SNP divergence, about .039, and (iii) carefully sustained, uniform, selection regimes featuring either B or C type conditions. We contend that this careful parallel replication makes the results of this Chapter notable with respect to its potential scientific salience, even though it is admittedly quite limited with respect to the total number of independent evolutionary replicates under test.

Evidently, we have tested for incipient reproductive isolation using stringent laboratory selection regimes of arbitrary design. There is nothing about the B or C culture conditions used here, or the populations that have adapted to those conditions, which warrants a claim that the present findings are faithful to any likely evolutionary scenario that might be exhibited by flies of the species *D. melanogaster.* This is in keeping with our general view that experimental evolution does not have scientific value primarily with respect to its close emulation of any particular set of circumstances in the wild (vid. Mueller et al. 2005). Instead, we would argue

that experimental evolution is at its best, as a scientific tool, when it is used to test very general scientific theories, especially theories that are not too content laden (vid. Rose et al. 2005; see also Garland and Rose 2009).

Of more importance, perhaps, the experimental populations that we have used to test the merits of null and ecological speciation mechanisms in Chapters Two and Three all feature moderately large effective population sizes in the range of 800 to 1200 (Mueller et al. 2013). With respect to the impact of severe or sustained population size bottlenecks, it is intuitively plausible that such reductions in population size should degrade environment-specific adaptation while fostering rare events of chromosomal rearrangement and the like (Rundle et al. 1998). In this respect, then, our results are probably biased against null mechanisms of speciation.

Alternative experimental strategies for testing ecological models of speciation

Unlike our suggestion at the end of Chapter Two with respect to fostering null speciation by reduced population sizes in experimental evolution paradigms, we do not think that ecological speciation is likely to be fostered in populations with significantly reduced population sizes. Rather, we would suggest that still larger population sizes in experimental populations might produce still greater levels of functional divergence, including perhaps female mating preferences more precisely attuned to the selection regimes imposed on them prior to tests for reproductive isolation. Put another way, ecological speciation depends primarily on functional differentiation, and functional differentiation is enhanced when selection is more powerful. Larger-population size experiments are likely to favor ecological speciation still more than our results do.

On the other hand, there is one experimental paradigm variation that is highly relevant, we would suggest. In this Chapter, we have studied incipient reproductive isolation arising from recently generated functional divergence in effective allopatry. For us, this raises the question as to whether or not the combination of functional divergence with long periods of sustained selection could produce still greater levels of reproductive isolation than we have found here. Chapter Four of this doctoral thesis is devoted to an experimental analysis of this very question.

Conclusion

Our overall conclusion is that we have presented strong evidence for the role of ecologically divergent selection among allopatric populations in producing incipient reproductive isolation, in this Chapter. With such ecologically divergent selection, over time periods of less than 200 generations, experimental evolution of Mendelian populations apparently produces a measureable degree of reproductive isolation. The following qualification should be born in mind, however. We have not found complete reproductive isolation, so we haven't demonstrated the sufficiency of ecologically divergent selection in speciation. The results of this Chapter were obtained only for one particular contrast of selection regime. These results were also obtained for moderately large populations that maintain a fair amount of genetic variation. Thus we have no direct basis for claiming that our results will be applicable to populations with much smaller or much larger effective population sizes. This Chapter does not explore the effects of longer-sustained periods of divergent ecological selection regimes during allopatry, which is the concern of the next Chapter of this thesis dissertation. On the other hand, we regard the present results as a dramatic corroboration of ecological speciation theory, using

an experimental paradigm comparable to those used to test null speciation theory, as described in the preceding Chapter. The contrasting results of these two Chapters, we believe, are striking and informative.

Acknowledgements

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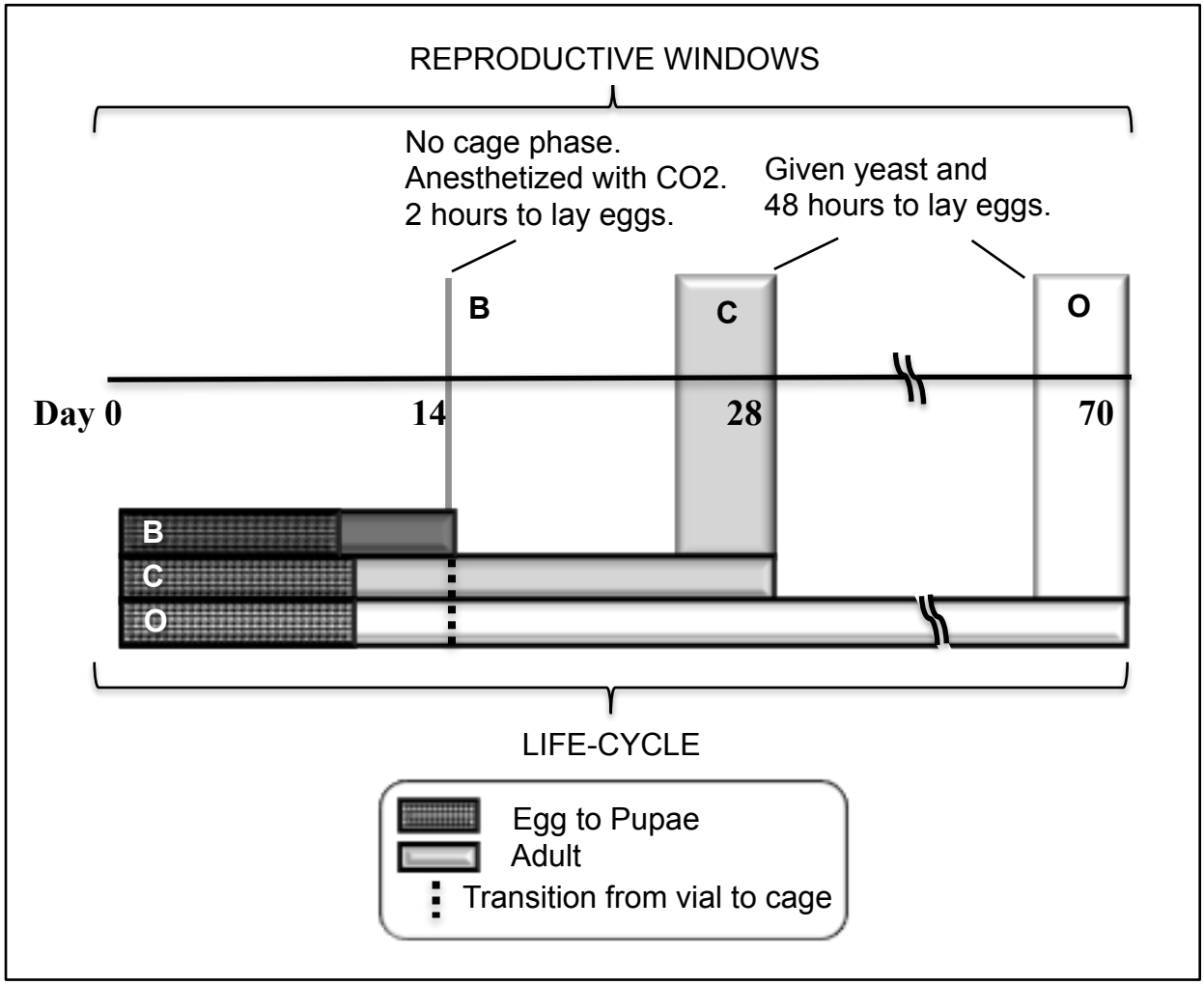

Figure 3.1. Distinct selection regimes imposed on five-fold replicated groups of outbred populations. The primary difference between selection regimes is the time interval (reproductive window) when eggs are collected to establish the next generation. Only **B**-type, and **C**-type populations were used in this study. Both the **B**O and n**C**O lineages were derived from the O lineage.

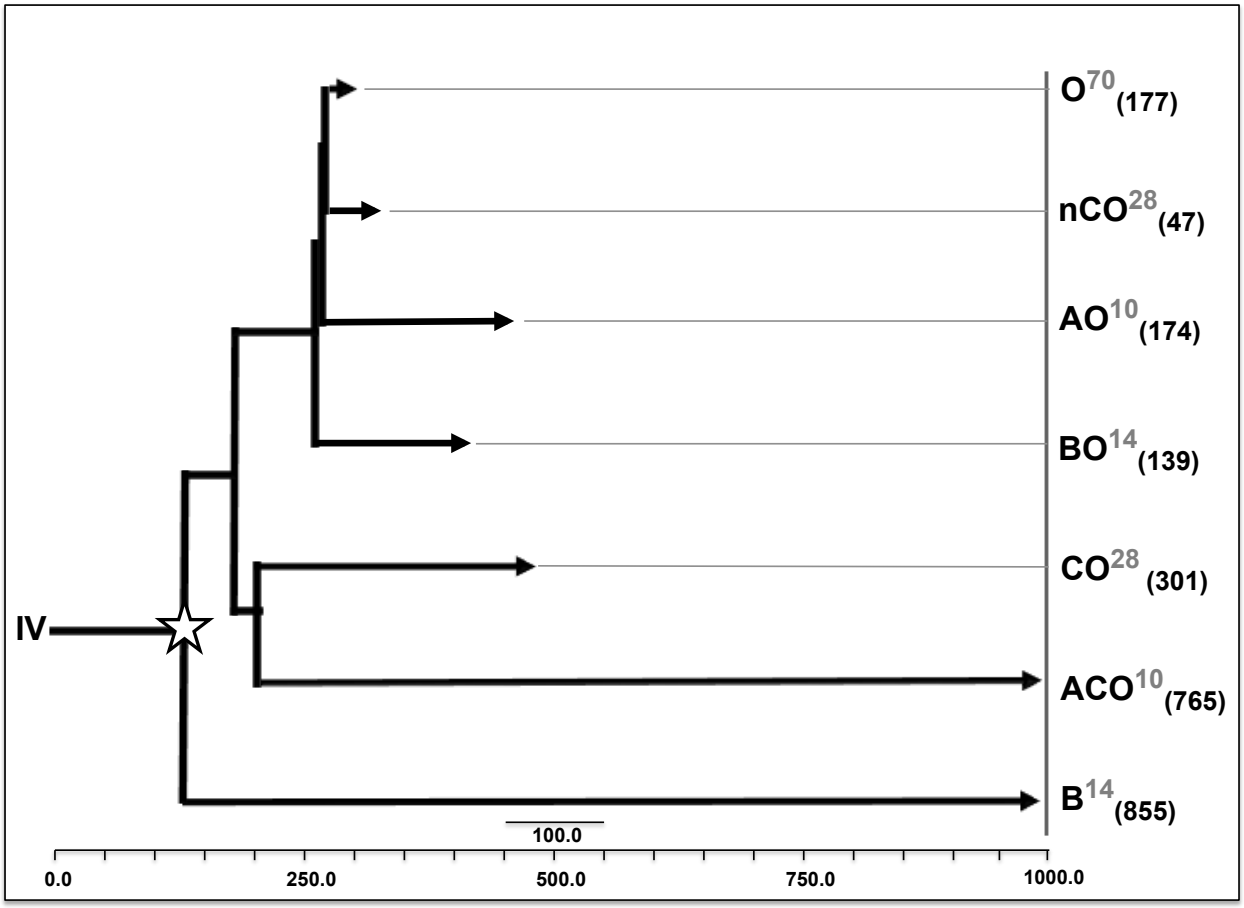

Figure 3.2. Collection of allopatric Drosophila laboratory populations derived from a single outbred population ("IV") in early1980 (star). Each selection regime was imposed on five populations. The X-axis gives the number of generations evolving under laboratory conditions. The Y-axis shifts indicate changes in selection regimes, with the life-cycle length of each selection regime indicated by the superscript, and the number generations evolving under distal selection regime indicated by the subscript.

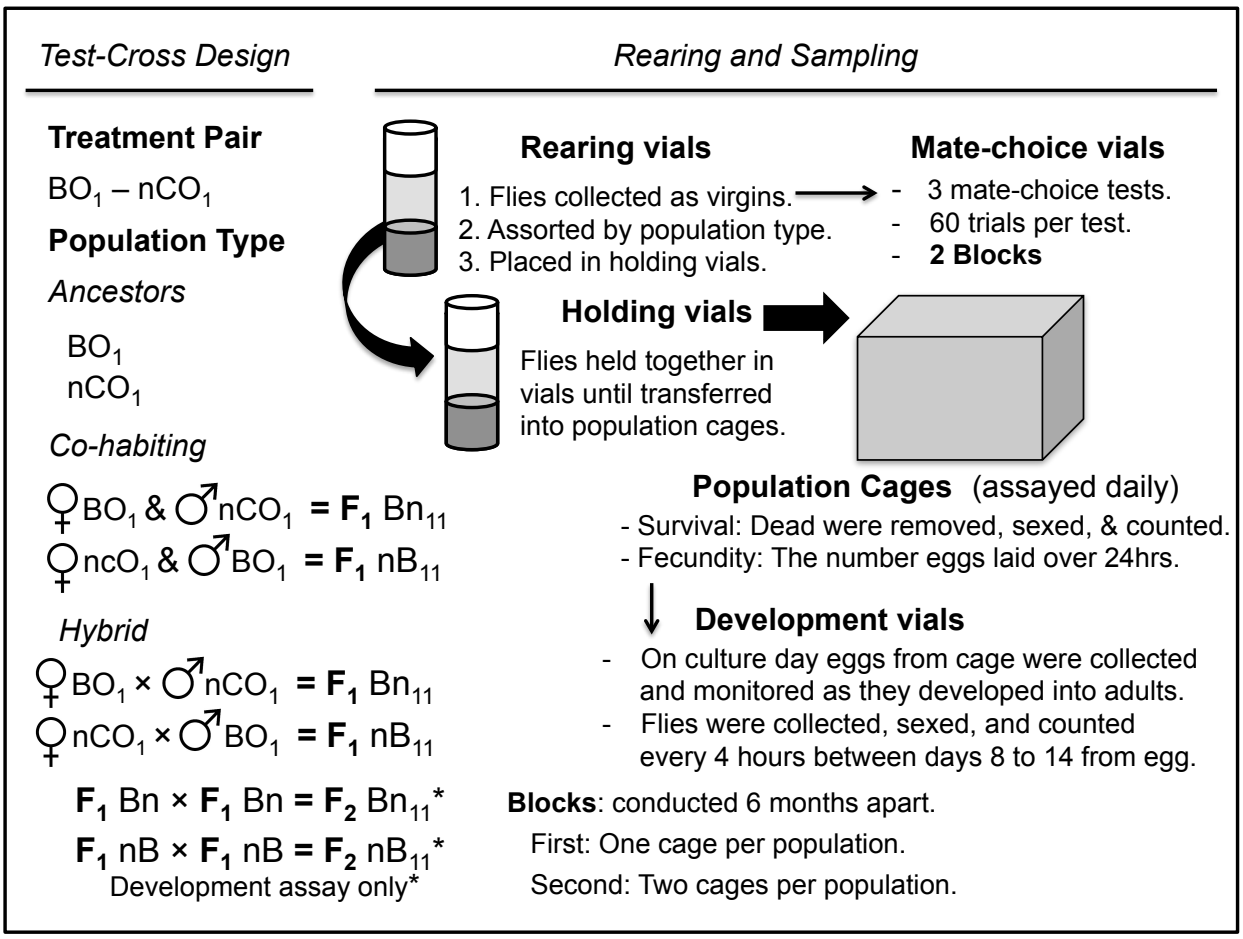

Figure 3.3. Overall experimental structure. There was a systematic experimental design difference between the two blocks. Single assay cohorts were used for each test-cross in block 1, while two assay cohorts were used for block 2.

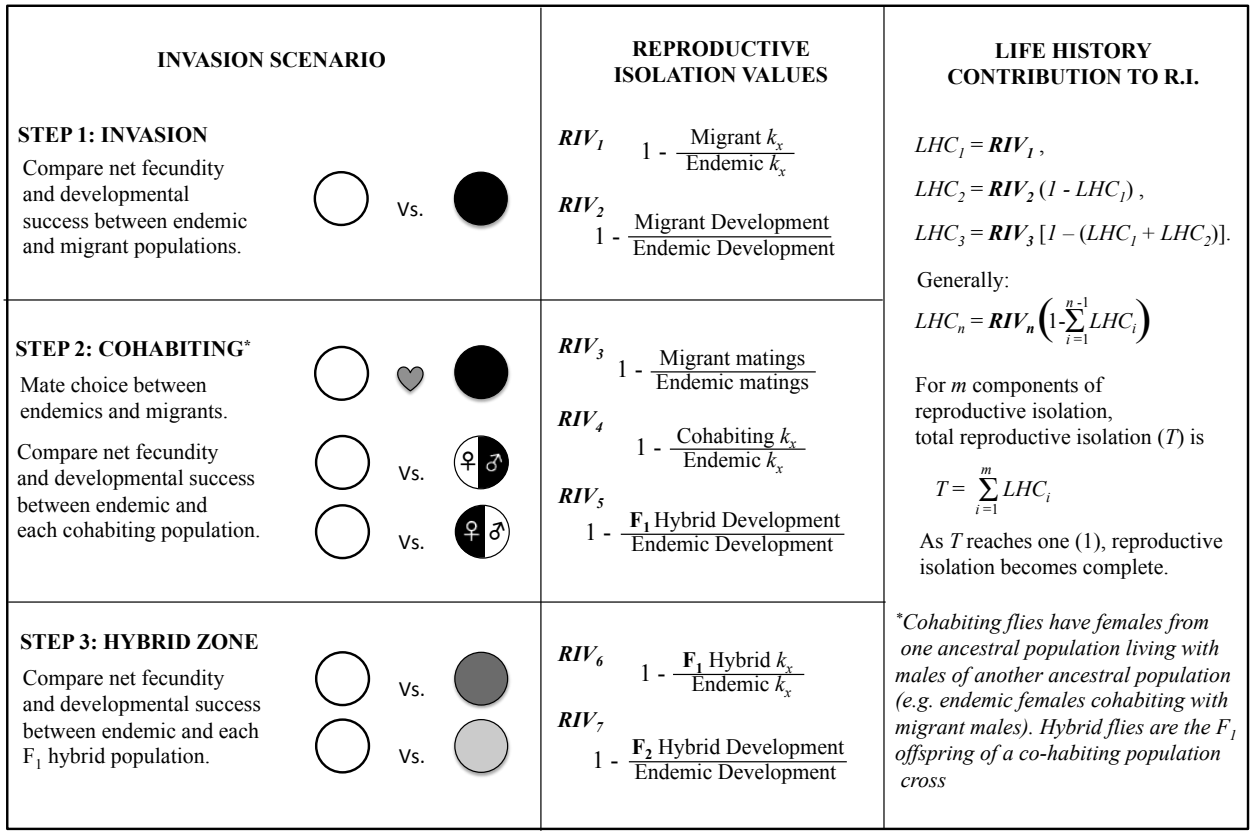

Figure 3.4. Schematic of a secondary contact scenario conceived as a model system for incipient reproductive isolation between long-allopatric populations. A composite measure of reproductive isolation was calculated using sequential life-history components.

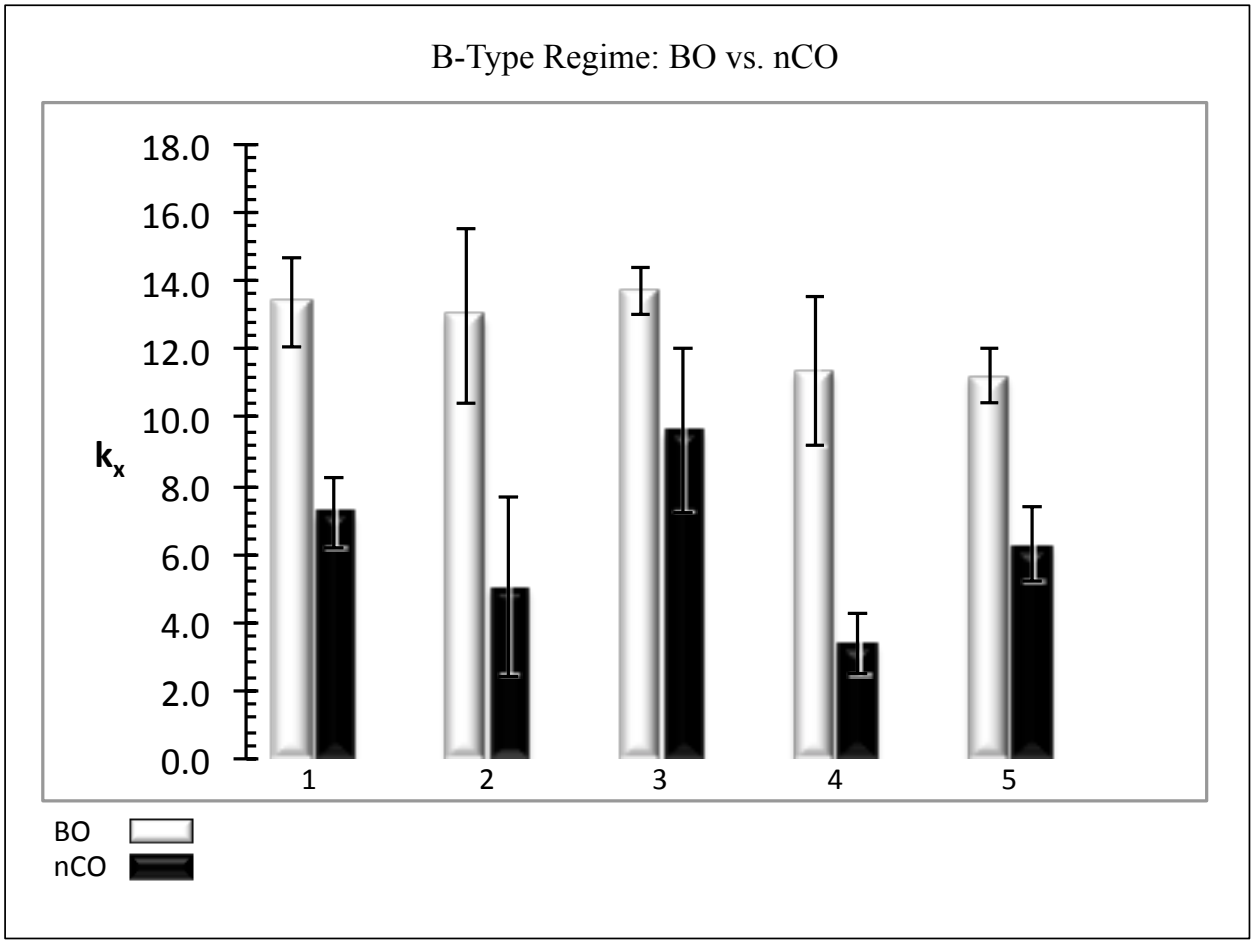

Figure 3.5. Focal net productivity comparison between the endemic BO and migrant nCO populations under B-type regime conditions. Presented is the average of the three observations of k_x per population independent of the block structure. Error bars represent standard error of the mean.

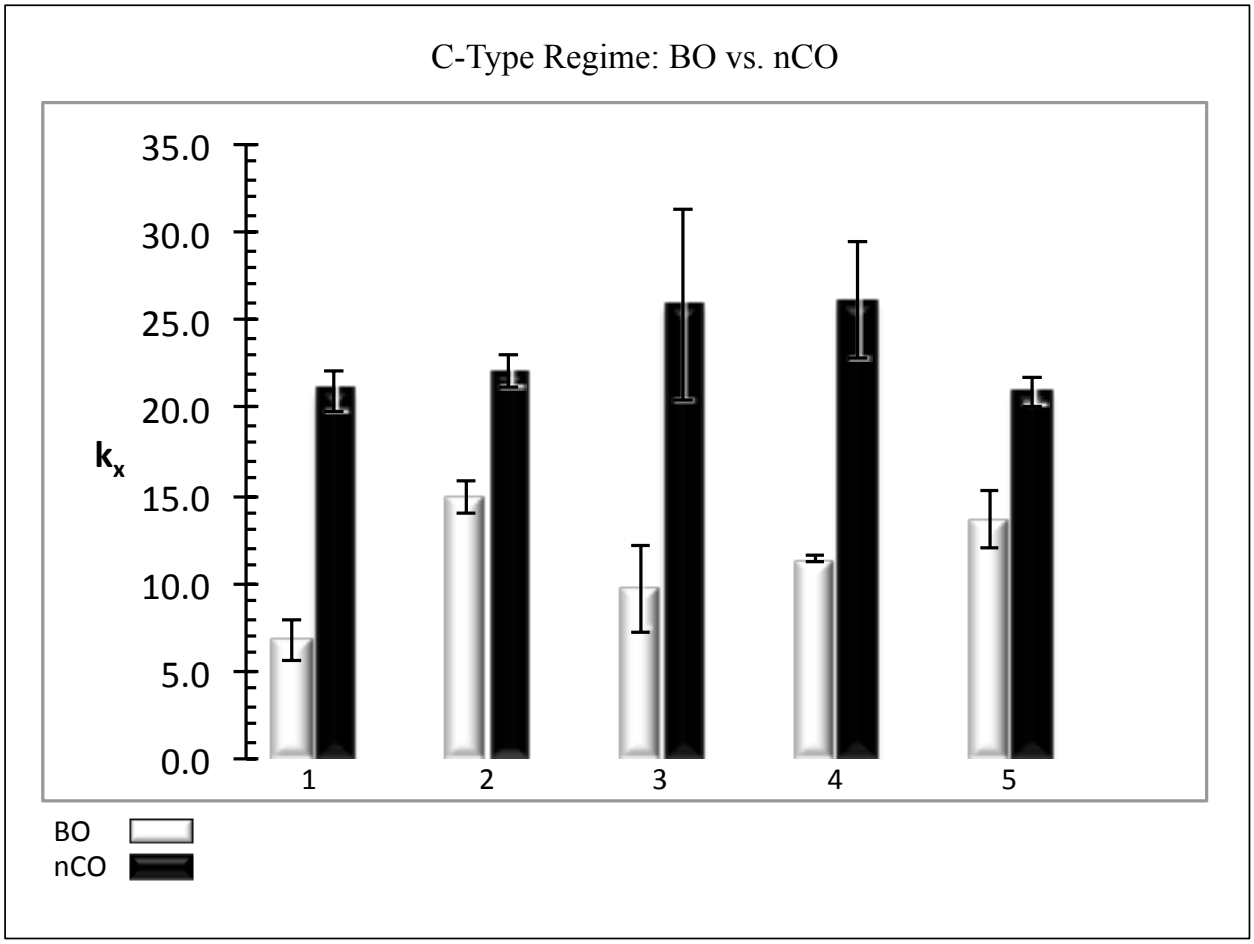

Figure 3.6. Focal net fecundity comparison between the migrant BO and endemic nCO populations under C-type regime conditions. Presented is the average of the three observations of k_x per population independent of the block structure. Error bars represent standard error of the mean.

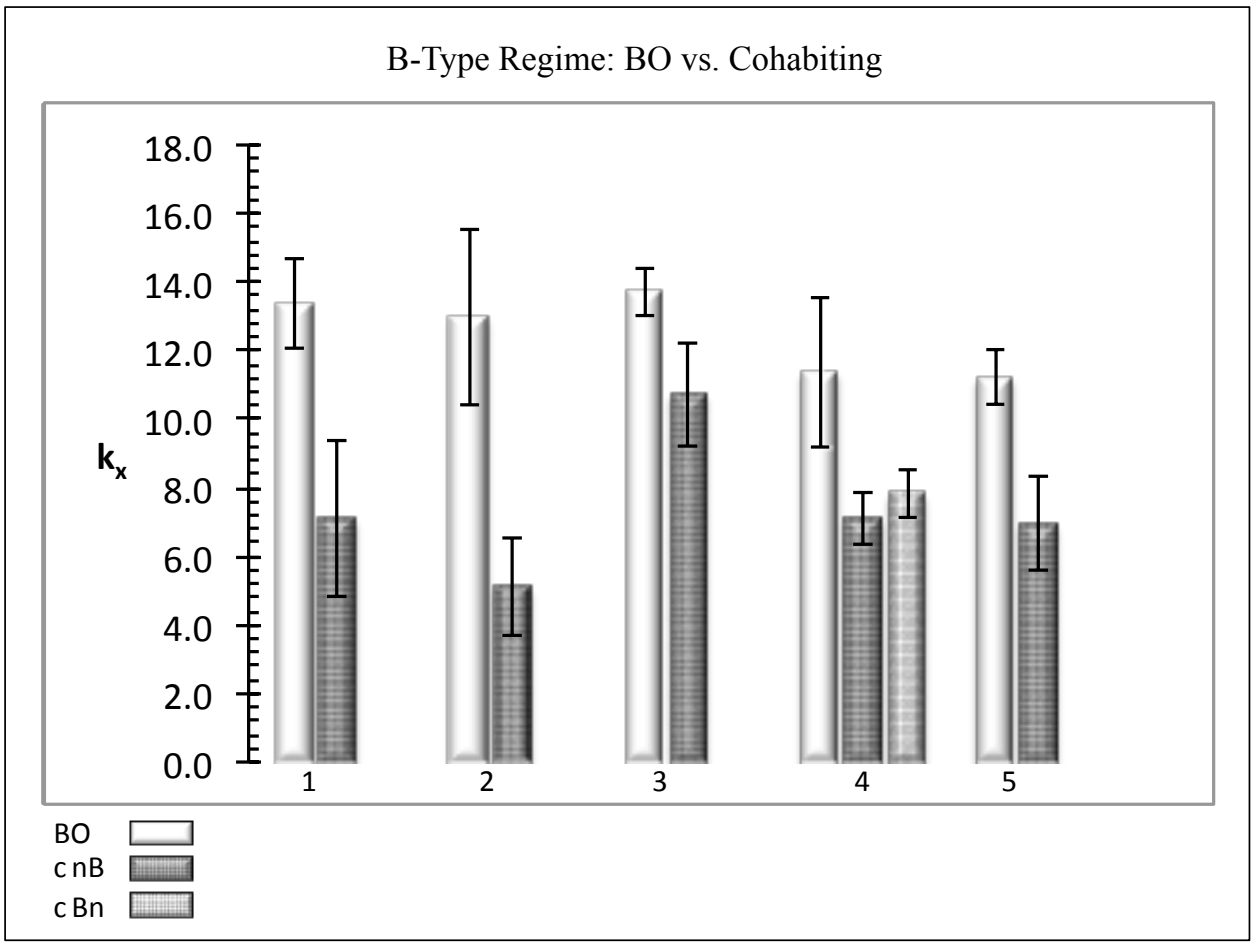

Figure 3.7. Focal net productivity comparison between the endemic BO populations and the Cohabiting population crosses under B-type regime conditions (c nB = nCO females with BO males; c Bn = BO females with nCO males). Presented is the average of the three observations of k_x per population independent of the block structure. Error bars represent standard error of the mean.

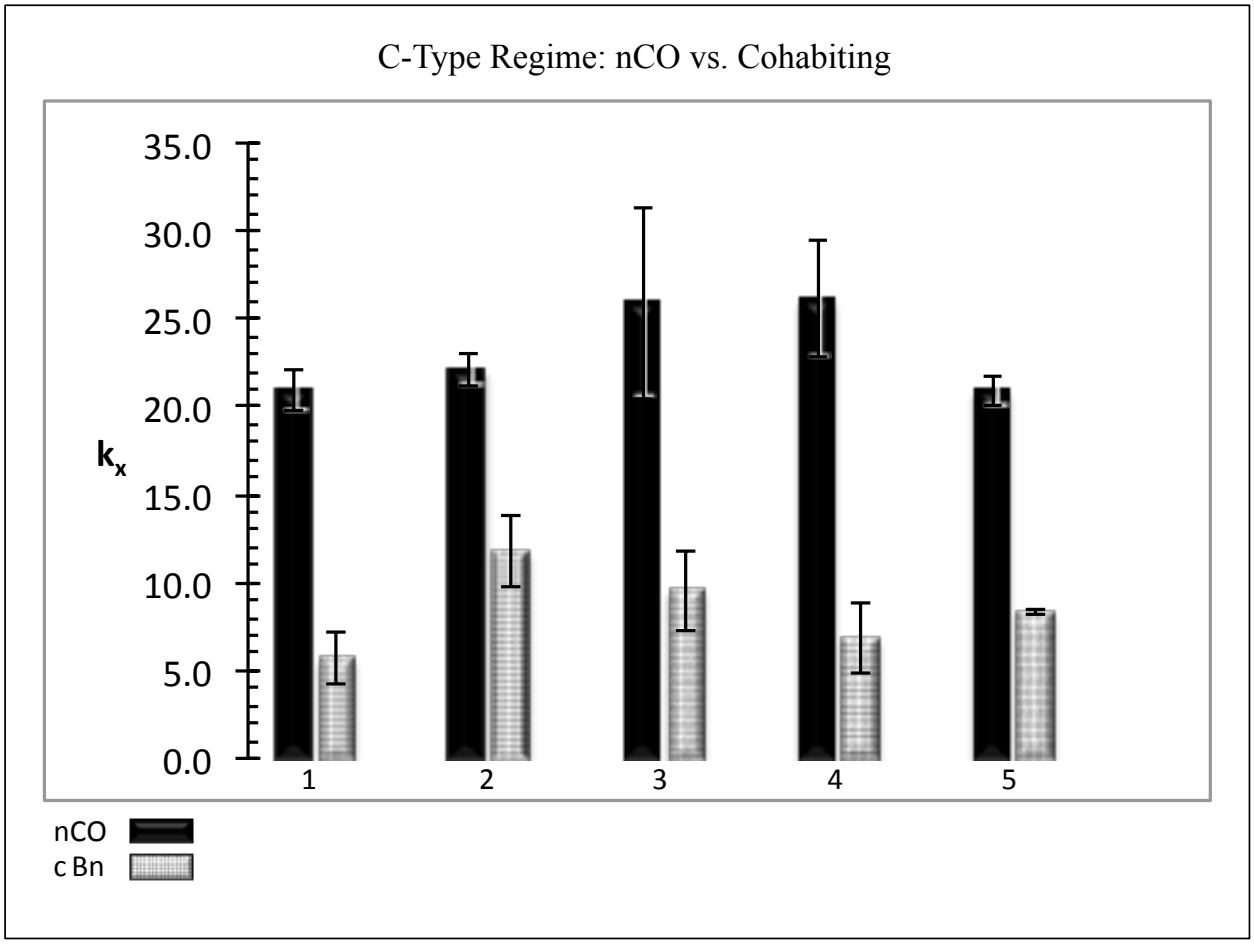

Figure 3.8. Focal net fecundity comparison between the endemic nCO populations and the Cohabiting population crosses under C-type regime conditions (c Bn = BO females with nCO males). Presented is the average of the three observations of k_x per population independent of the block structure. Error bars represent standard error of the mean.

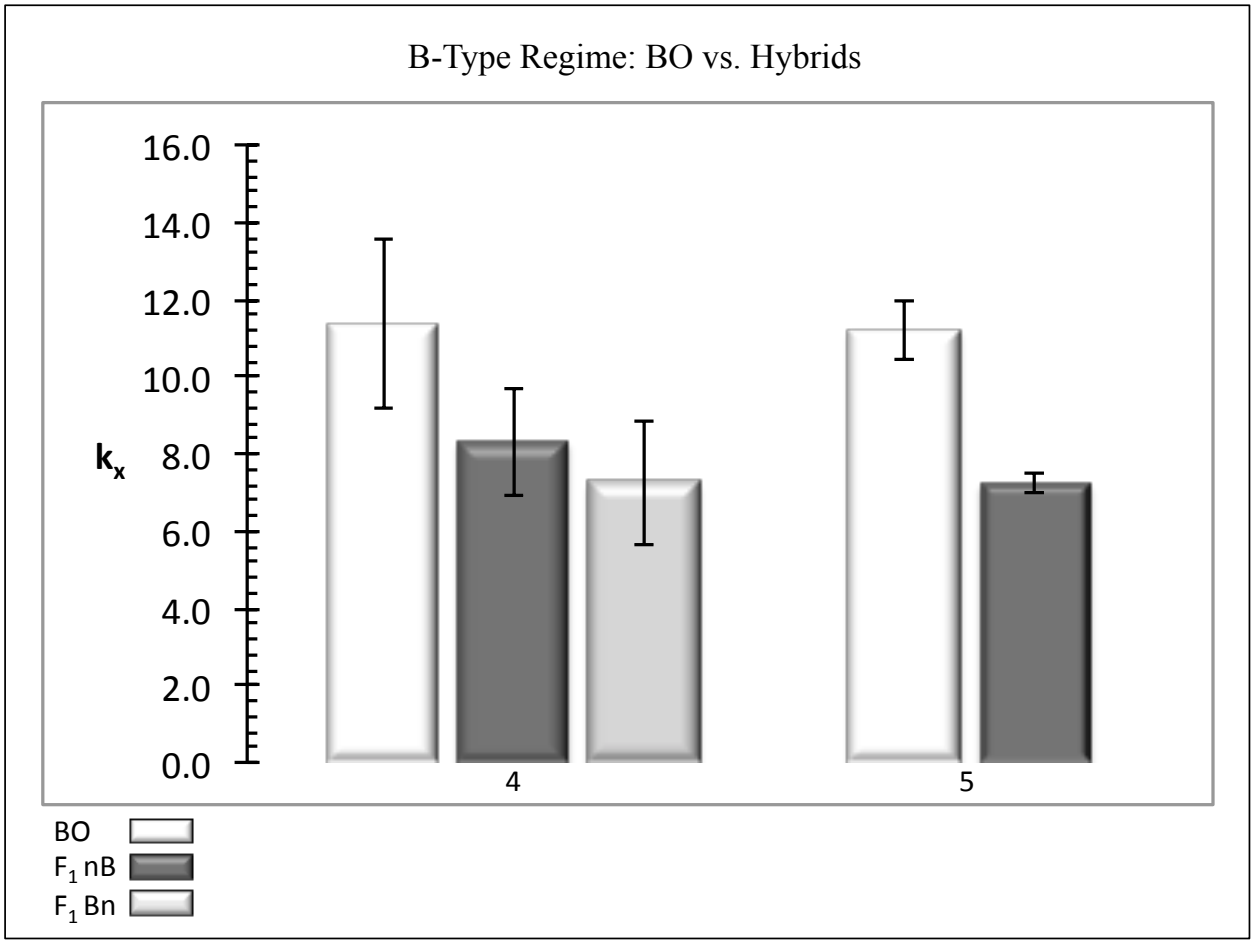

Figure 3.9. Focal net productivity comparison between the endemic BO populations and the F_1 hybrid populations under B-type regime conditions. Presented is the average of the three observations of k_x per population independent of the block structure. Error bars represent standard error of the mean.

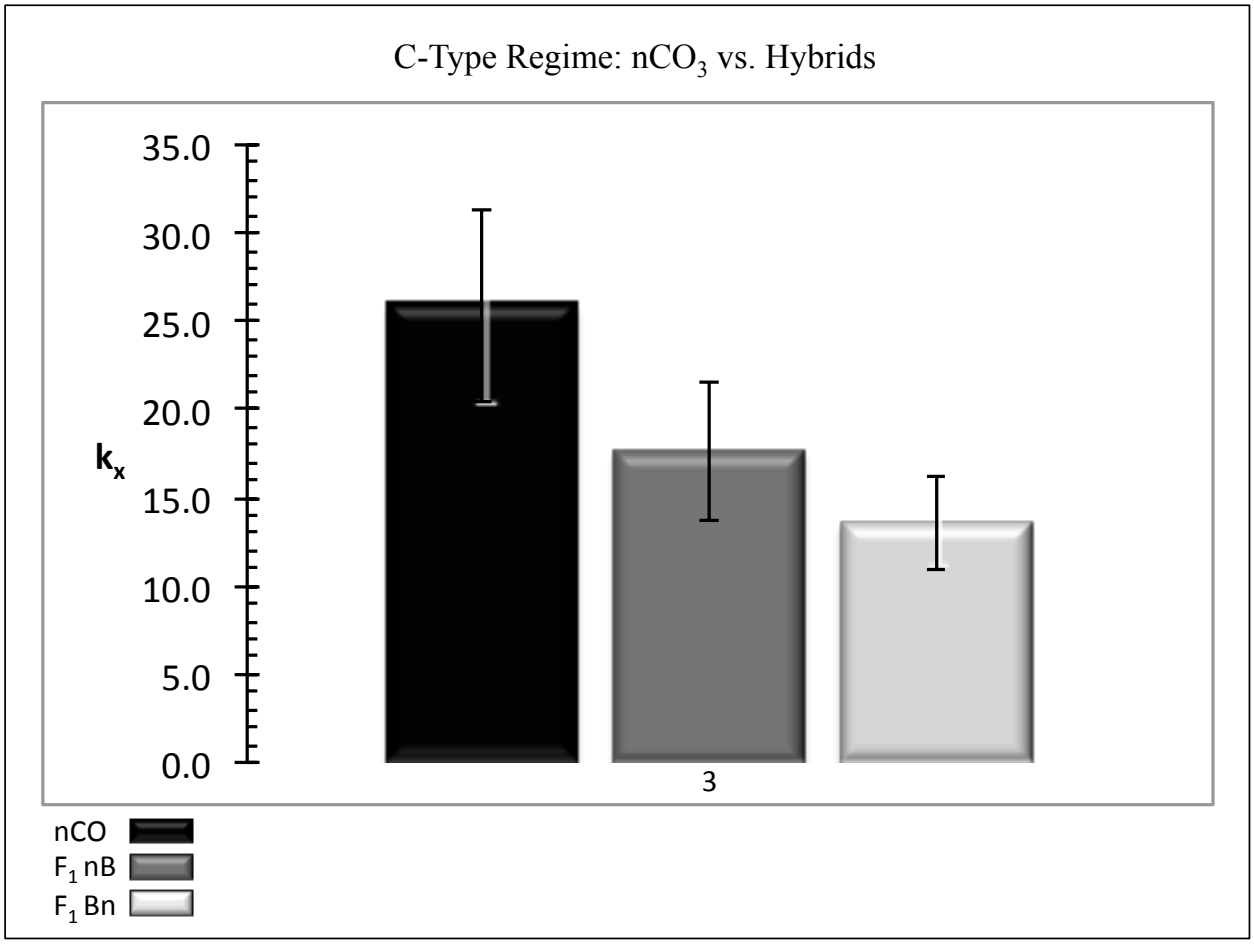

Figure 3.10. Focal net fecundity comparison between the endemic nCO_3 and the F_1 hybrid populations under C-type regime conditions. Presented is the average of the three observations of k_x per population independent of the block structure. Error bars represent standard error of the mean.

Table 3.1. Components of reproductive isolation for test-crosses under the B-type culture-regime environment. Isolation components vary from zero (no barrier) to one (complete isolation). Negative component values indicate life-history stages at which hybridization is favored. Each RI-value is reported as the average between blocks. Asterisks indicate p-values below .05.

	nCO_i invades BO_i					
Pop. Cross R.I. Barrier	Pop 1.	R.I.V.	*p*-value	Pop 2.	R.I.V.	*p*-value
nCO_1èBO_1						
Ancestor k_x	nCO_1	0.412 ± .11	0.031*	BO_1	-	-
Mate-choice	nCO_1 (F)	-0.236 ± .04	0.261	BO_1 (F)	0.359 ± .02	0.022*
Cohabiting k_x	c nB_{11}	0.309 ± .36	0.026*	c Bn_{11}	0.045 ± .03	0.999
F_1 Hybrid k_x	F_1nB_{11}	-0.001 ± .24	0.981	F_1Bn_{11}	-0.011 ± .25	0.987
Total						
nCO_2èBO_2						
Ancestor k_x	nCO_2	0.598 ± .17	<.0001*	BO_2	-	-
Mate-choice	nCO_2 (F)	0.114 ± .16	0.428	BO_2 (F)	0.232 ± .05	0.162
Cohabiting k_x	c nB_{22}	0.605 ± .05	<.0001*	c Bn_{22}	0.076 ± .18	0.997
F_1 Hybrid k_x	F_1nB_{22}	0.338 ± .21	0.068	F_1Bn_{22}	0.320 ± .06	0.151
Total						
nCO_3èBO_3						
Ancestor k_x	nCO_3	0.388 ± .24	.0004*	BO_3	-	-
Mate-choice	nCO_3 (F)	-0.369 ± .44	0.158	BO_3 (F)	0.318 ± .11	0.071
Cohabiting k_x	c nB_{33}	0.266 ± .13	0.028*	c Bn_{33}	0.166 ± .08	0.396
F_1 Hybrid k_x	F_1nB_{33}	0.153 ± .09	0.551	F_1Bn_{33}	0.125 ± .10	0.825
Total						
nCO_4èBO_4						
Ancestor k_x	nCO_4	0.713 ± .02	<.0001*	BO_4	-	-
Mate-choice	nCO_4 (F)	0.162 ± .23	0.380	BO_4 (F)	0.042 ± .25	0.549
Cohabiting k_x	c nB_{44}	0.295 ± .14	.0005*	c Bn_{44}	0.225 ± .15	0.008*
F_1 Hybrid k_x	F_1nB_{44}	0.236 ± .05	0.037*	F_1Bn_{44}	0.386 ± .04	0.001*
Total						
nCO_5èBO_5						
Ancestor k_x	nCO_5	0.421 ± .07	<.0001*	BO_5	-	-
Mate-choice	nCO_5 (F)	-0.095 ± .10	0.689	BO_5 (F)	0.218 ± .18	0.162
Cohabiting k_x	c nB_{55}	0.356 ± .10	<.0001*	c Bn_{55}	0.129 ± .01	0.452
F_1 Hybrid k_x	F_1nB_{55}	0.375 ± .09	<.0001*	F_1Bn_{55}	0.102 ± .02	0.657
Total						

Table 3.2. Components of reproductive isolation for test-crosses under the C-type culture-regime environment. Isolation components vary from zero (no barrier) to one (complete isolation). Negative component values indicate life-history stages at which hybridization is favored. Each RI-value is reported as the average between blocks. Asterisks indicate p-values below .05.

	BO_i invades nCO_i					
Pop. Cross R.I. Barrier	Pop 1.	R.I.V.	*p*-value	Pop 2.	R.I.V.	*p*-value
BO_1ènCO_1						
Ancestor k_x	BO_1	0.703 ± .09	<.0001*	nCO_1	-	-
Mate-choice	BO_1 (F)	0.359 ± .02	0.022*	nCO_1 (F)	-0.236 ± .04	0.261
Cohabiting k_x	c Bn_{11}	0.724 ± .02	<.0001*	c nB_{11}	0.056 ± .18	1.000
F_1 Hybrid k_x	F_1Bn_{11}	0.314 ± .03	0.196	F_1nB_{11}	0.389 ± .27	0.163
Total						
BO_2ènCO_2						
Ancestor k_x	BO_2	0.338 ± .03	0.010*	nCO_2	-	-
Mate-choice	BO_2 (F)	0.232 ± .05	0.162	nCO_2 (F)	0.114 ± .16	0.428
Cohabiting k_x	c Bn_{22}	0.508 ± .11	<.0001*	c nB_{22}	-0.089 ± .17	0.613
F_1 Hybrid k_x	F_1Bn_{22}	0.126 ± .12	0.957	F_1nB_{22}	0.164 ± .09	0.765
Total						
BO_3ènCO_3						
Ancestor k_x	BO_3	0.629 ± .00	<.0001*	nCO_3	-	-
Mate-choice	BO_3 (F)	0.318 ± .11	0.071	nCO_3 (F)	-0.369 ± .44	0.158
Cohabiting k_x	c Bn_{33}	0.648 ± .03	<.0001*	c nB_{33}	0.065 ± .08	0.836
F_1 Hybrid k_x	F_1Bn_{33}	0.413 ± .10	<.0001*	F_1nB_{33}	0.261 ± .10	0.008*
Total						
BO_4ènCO_4						
Ancestor k_x	BO_4	0.517 ± .09	<.0001*	nCO_4	-	-
Mate-choice	BO_4 (F)	0.042 ± .25	0.549	nCO_4 (F)	0.162 ± .23	0.380
Cohabiting k_x	c Bn_{44}	0.667 ± .14	<.0001*	c nB_{44}	-0.001 ± .03	1.000
F_1 Hybrid k_x	F_1Bn_{44}	0.271 ± .04	0.136	F_1nB_{44}	0.263 ± .11	0.079
Total						
BO_5ènCO_5						
Ancestor k_x	BO_5	0.383 ± .12	0.029*	nCO_5	-	-
Mate-choice	BO_5 (F)	0.218 ± .18	0.162	nCO_5 (F)	-0.095 ± .10	0.689
Cohabiting k_x	c Bn_{55}	0.604 ± .01	<.0001*	c nB_{55}	-0.021 ± .11	0.997
F_1 Hybrid k_x	F_1Bn_{55}	0.373 ± .17	0.054	F_1nB_{55}	0.069 ± .28	1.000
Total						

Table 3.3. Summary of reproductive isolation for test-crosses under the B-type culture-regime environments. Isolation components vary from zero (no barrier) to one (complete isolation). Negative component values indicate life-history stages at which hybridization is favored. Part A lists life history contribution to reproductive isolation calculated with RI-values regardless of statistical significance. Part B lists life history contribution to reproductive isolation calculated with only RI-values that are statistically significant.

nCO_i invades BO_i: Life History Contribution to Reproductive Isolation

Average R.I.	R.I. Barrier	Replicates				
		1 × 1	2 × 2	3 × 3	4 × 4	5 × 5
A)	Ancestor k_x	0.412	0.598	0.388	0.713	0.421
All R.I. Values	Mate-choice	0.033	0.079	0.049	0.029	0.032
0.703 ± .07	Cohabiting k_x	0.093	0.123	0.123	0.069	0.141
	F_1 Hybrid k_x	-0.056	0.062	0.051	0.057	0.099
	Total	0.482	0.862	0.611	0.868	0.694
B)	Ancestor k_x	0.412	0.598	0.388	0.713	0.421
Stat. Sig. R.I.V.	Mate-choice	0.105	0.000	0.000	0.000	0.000
0.642 ± .07	Cohabiting k_x	0.071	0.117	0.066	0.077	0.099
	F_1 Hybrid k_x	0.000	0.000	0.000	0.066	0.076
	Total	0.588	0.715	0.454	0.856	0.596

Table 3.4. Summary of reproductive isolation for test-crosses under the C-type culture-regime environments. Isolation components vary from zero (no barrier) to one (complete isolation). Negative component values indicate life-history stages at which hybridization is favored. Part A lists life history contribution to reproductive isolation calculated with RI-values regardless of statistical significance. Part B lists life history contribution to reproductive isolation calculated with only RI-values that are statistically significant.

BO_i invades nCO_i: Life History Contribution to Reproductive Isolation

Average R.I.	R.I. Barrier	Replicates				
		1 × 1	2 × 2	3 × 3	4 × 4	5 × 5
A)	Ancestor k_x	0.703	0.338	0.629	0.517	0.383
All R.I. Values	Mate-choice	0.021	0.113	-0.008	0.050	0.043
0.717 ± .06	Cohabiting k_x	0.070	0.092	0.093	0.152	0.122
	F_1 Hybrid k_x	0.057	0.056	0.072	0.064	0.022
	Total	0.850	0.599	0.785	0.782	0.570
B)	Ancestor k_x	0.703	0.338	0.629	0.517	0.383
Stat. Sig. R.I.V.	Mate-choice	0.054	0.000	0.000	0.000	0.000
0.679 ± .07	Cohabiting k_x	0.069	0.167	0.120	0.155	0.186
	F_1 Hybrid k_x	0.000	0.000	0.076	0.000	0.000
	Total	0.826	0.505	0.825	0.671	0.569

Table 3.5. Results of the mate-choice assay for prezygotic reproductive isolation between BO and nCO populations. Asterisks indicate p-values below .05.

Populations	Female	Male 1	Male 2	Matings		*p*-value		
				Male 1	Male 2	Block 1	Block 2	Both
BO_1 vs. nCO_1	BO_1	BO_1	nCO_1	67	43	0.115	0.096	0.022*
	nCO_1	nCO_1	BO_1	51	63	0.354	0.508	0.261
BO_2 vs. nCO_2	BO_2	BO_2	nCO_2	65	50	0.438	0.225	0.162
	nCO_2	nCO_2	BO_2	55	47	0.233	0.881	0.428
BO_3 vs. nCO_3	BO_3	BO_3	nCO_3	59	41	0.362	0.086	0.072
	nCO_3	nCO_3	BO_3	49	64	0.027	0.785	0.158
BO_4 vs. nCO_4	BO_4	BO_4	nCO_4	53	47	0.189	0.537	0.549
	nCO_4	nCO_4	BO_4	57	48	0.793	0.101	0.38
BO_5 vs. nCO_5	BO_5	BO_5	nCO_5	57	43	0.884	0.074	0.162
	nCO_5	nCO_5	BO_5	48	52	1.000	0.555	0.689

Table 3.6. Summary of total reproductive isolation for BO and nCO test-crosses. Isolation components vary from zero (no barrier) to one (complete isolation). Negative component values indicate life-history stages at which hybridization is favored. Part A lists life history contribution to reproductive isolation calculated with RI-values regardless of statistical significance. Part B lists life history contribution to reproductive isolation calculated with only RI-values that are statistically significant.

Total Life History Contribution to Reproductive Isolation

Invasion	Replicates					Ave. R.I.
	1 × 1	2 × 2	3 × 3	4 × 4	5 × 5	
A) All R.I. Values						
BO*i* è nCO*i*	0.850	0.599	0.785	0.782	0.570	0.717 ± .06
nCO*i* è BO*i*	0.482	0.862	0.611	0.868	0.694	0.703 ± .07
R.I. Between Pops.	0.666	0.731	0.698	0.825	0.632	0.710 ± .03
B) Stat. Sig. R.I. Values						
BO*i* è nCO*i*	0.826	0.505	0.825	0.671	0.569	0.679 ± .07
nCO*i* è BO*i*	0.588	0.715	0.454	0.856	0.596	0.642 ± .07
R.I. Between Pops.	0.707	0.610	0.640	0.764	0.583	0.661 ± .03

CHAPTER 4

Testing for an Interaction Between
Ecological Selection and other Evolutionary Mechanisms In Speciation

Abstract

Since Darwin published the *Origin of Species*, biologists have contended that divergent natural selection is a common force in creating reproductive isolation. Others have disagreed, arguing for other evolutionary mechanisms instead. We used replicate outbred populations of *Drosophila melanogaster* that have been experimentally evolved for hundreds of generations under contrasting as well as parallel selection regimes, in order to test the importance of divergent selection compared to other evolutionary mechanisms in initiating reproductive isolation between allopatric populations. We tested two groups of *D. melanogaster* populations, each with ten replicate populations. These two groups differ in development time (egg to adult), with one developing rapidly and the other developing slowly. There is extensive genome-wide differentiation both between and within these groups of populations, according to analysis of their single nucleotide polymorphisms (SNP). Parental populations and their crosses were phenotypically assayed for the following characters: (1) mate-choice, (2) mortality, (3) fecundity, and (4) developmental success. We found little significant evidence of reproductive isolation among populations *within* each of these two groups, populations that have evolved in parallel for hundreds of generations with the potential for significant evolutionary divergence by mechanisms other than divergent selection. By contrast, we found strong evidence for incipient reproductive isolation in crosses *between* groups of divergently selected populations. In addition, we did not find a statistically greater signal of reproductive isolation when the populations involved in any of these crosses had a greater time since their last shared common ancestor. This result suggests that there is no detectable interaction of time and selective differentiation in initiating reproductive isolation, at least in populations that have not been subjected to inbreeding. Overall, our conclusion is that differences in selection regime have

greater relative importance than evolutionary time in fostering reproductive isolation between allopatric populations.

Introduction

Implicit in Darwin's (1859) original theory of evolution by natural selection was the hypothesis that phenotypic divergence produced by directional selection would in turn lead to speciation, speciation being crudely conceived by Darwin as the process whereby evolutionary lineages become separate. Naturally, the thinking of evolutionary biologists on this point was not entirely clear in the 19th Century. During the first half of the 20th Century, evolutionary biology incorporated genetics, and the discussion of speciation was progressively clarified (e.g. Dobzhansky, 1937; "Genetics and the Origin of Species," 1st Edition).

A number of late 20th Century biologists developed a different view from Darwin's about the role of natural selection in speciation, proposing hypothetical speciation scenarios featuring little or no role for ecological adaptation in speciation (e.g. Rose and Doolittle 1983). There are many such "non-ecological" speciation hypotheses, too many to be usefully reviewed here, although we should be clear that this broad category includes mutation-order speciation (Mani and Clarke 1990), speciation by genetic drift (Lande 1981), and polyploidy speciation (Winge 1917). Recent synoptic reviews of such speciation scenarios have been provided by Schluter (2001), Turelli et al. (2001), Coyne and Orr (2004), Schluter (2009), and Sobel et al. (2009). Here we refer to all such hypotheses collectively as constituting a "null" theory or model for speciation, in order to clearly differentiate them from those which follow Darwin's lead. As will be shown below, not only is this a useful theoretical distinction (vid. Schluter 2000, 2001;

Rundle and Nosil 2005; Schluter 2009, Nosil et al. 2009), it can also be an experimentally practical categorization.

In recent years, however, a more specifically Darwinian view has been brought to the fore by Schluter (e.g. 2009), Via (e.g. 2009) and others (Funk 1998; Rundle and Nosil 2005; reviewed in Coyne and Orr 2004). Here we will refer to this view as its proponents often do as the "ecological speciation hypothesis," which we take as the theory that the evolution of reproductive isolation between populations is chiefly underlain by divergent phenotypic adaptation in response to natural selection arising from differences between environments. It implies that reproductive isolation should be more likely to evolve between allopatric populations that have phenotypically adapted to different environments, compared to allopatric populations that have adapted to similar environments by evolving similar phenotypes.

In order to juxtapose ecological speciation and null speciation models, the distinction between the two can be defined according to whether or not divergent phenotypic adaptation plays a role in reproductive isolation. Two such broadly distinct explanations for speciation might seem to afford few opportunities for critical hypothesis tests, but here we endeavor to demonstrate that our laboratory can in fact perform strong-inference comparisons of these two major theoretical alternatives for speciation.

Key to our testing of these hypotheses is a collection of long-established laboratory populations that has the following features. First, we have multiply replicated populations that have evolved in parallel in similar environments, such that only candidate null speciation scenarios are likely to be responsible for their incipient reproductive isolation, if it arises in such cases. Second, we have multiple sets of replicated populations that have undergone contrasting selection regimes that have led to considerable, functional, phenotypic divergence.

Third, there is evidence that some of these populations are in the initial stages of evolving reproductive isolation.

Because of the scale and replication of the Rose laboratory system of experimentally evolved sexual populations, we are in a position to address the relative merits of the alternative speciation scenarios *quantitatively,* given the large number of possible crosses within and between differentiated groups of replicated populations that do and do not share common selective regimes. Moreover, as these populations have widely varied numbers of generations since they last shared a common ancestor, the importance of number of generations of evolutionary independence for the evolution of reproductive isolation can be quantitatively evaluated.

We are not looking for individual corroborative or falsifying instances. Rather, our objective is *to test alternative speciation hypotheses statistically across an ensemble of populations*. Specifically, we are testing whether incipient reproductive isolation is more common among populations that have phenotypically diverged due to selection, compared to its incidence among populations that have been maintained under identical ecological regimes, but have evolved separately from each other for many generations.

In our laboratory, we have dozens of populations of *D. melanogaster* undergoing selection, with five or six replicate populations maintained for each selection regime. The estimated effective population size for each population is about 1000 (Mueller et al., 2013). For the 30 populations that we are particularly interested in for this proposal, their selection treatments differ chiefly with respect to the length of their discrete generations: the duration from egg-collection that starts each generation to the egg-laying that starts the next generation.

Taken together, these replicated treatment-groups constitute lineages that have been sustained for as long as 1,100 generations in our hands.

There is independent evidence of incipient reproductive isolation among replicate populations from the B group of populations created in 1980 (vid. Rose, 1984). Long et al. (2006) performed reciprocal crosses between the six IV and B populations, which had been maintained under identical conditions for 637 generations at the time of their experiments. They found seven of the 30 crosses with 'foreign' mates resulted in significant reductions in female components of fitness, whereas two resulted in significant increases in female components of fitness, compared to 'endemic' matings.

In the experiments presented in Chapters 2 and 3, we reported on crosses that give the greatest signals for the action of either null or ecological speciation mechanisms in the evolution of our laboratory populations. But the natural question that follows on from the hypothesis tests of those first two phases of our work is whether or not there are significant interactions between these two potential contributors to reproductive incompatibility. Darwin repeatedly emphasized that natural selection acts over long periods of time (Zimmer 2006; Reznick 2012). With evolution on a sufficiently long time-scale, it is likely that allopatric populations will undergo periods of parallel selection as well as periods in which ecological differences establish contrasting selection regimes. This raises the question whether longer-term evolution could produce reproductive isolation as a result of both ecological divergence and "null" sources of differentiation, such as genetic drift, transposition, and structural rearrangements that are not related to ecological selection mechanisms. Scenarios that combine ecological selection for differentiation with protracted opportunities for non-ecological genetic divergence we will call the "Interaction " speciation hypothesis here. Our point is to differentiate the abrupt impact of

divergent ecological selection on reproductive isolation, as instantiated in Chapter 3, from evolutionary scenarios that *add* to such selection prolonged periods of allopatry in which other evolutionary genetic mechanisms might act.

Fortunately, our experimental evolution phylogeny of laboratory populations naturally allows some very powerful combinations of crosses for addressing this question, because we can readily combine wide disparities in *both* ancestry and phenotypic differentiation among crosses.

Experimental Overview

We are testing three potential underlying causes of reproductive isolation: (1) the number of generations between populations following an evolutionary arc through a common ancestor; (2) the terminal treatment difference between populations; and finally (3) the interaction between these two effects.

In Chapter 2, we conducted three independent sets of tests for incipient reproductive isolation between populations that had evolved in allopatry under identical conditions for hundreds of generations. We found little evidence for an effect of numerous generations in allopatry on reproductive isolation for any life history character. We did, however, find some evidence for minor hybrid vigor effects. In Chapter 3, we tested for reproductive isolation between populations that had evolved under different selection regimes, with a much smaller number of generations since their last common ancestor compared to most of the populations tested in Chapter 2. We found strong evidence for the effect of last selection regime on reproductive isolation across multiple life history characters in the experiments of Chapter 3.

In this Chapter 4, we test for synergistic interactions between duration of allopatry and recent functional differentiation on reproductive isolation, using crosses that vary in both

ancestry and functional differentiation. For example, the ACO populations are five derivatives of the CO populations, each ACO_i population deriving from each CO_i ancestral line, which in turn are derived from each O_i. But note that an ACO_j replicate has a much greater number of generations separating it from CO_i ($i \neq j$) than ACO_i has separating it from CO_i, because the ACO_i and CO_i populations derived from the O_i population were not subject to the additional 400 generations of evolutionary divergence that separates different O lines, which last had a common ancestor in 1980. To this end, we performed some crosses among the ACO and CO replicate lines, where $i \neq j$, as shown in Figure 4.1.

The salience of these particular crosses for "Interaction" speciation is that they combine protracted periods of parallel selection with periods of divergent ecological selection. If there is no interaction between null and ecological speciation mechanisms, then the best statistical fit to the data will not include interactions between generation number and phenotypic divergence. That is, such a result would show that the two basic kinds of speciation mechanism, null and ecological, act independently. But if we don't get such a result, if instead there *are* such interactions, we will have shown that they can interact with each other in establishing incipient reproductive isolation in a well-defined system.

Materials and Methods

Experimental Populations

Experimental evolutionary history: This study uses outbred lab populations of *Drosophila melanogaster* selected for different patterns of age-specific reproduction. All the flies used in this study ultimately originate from an ancestral "IV" population first collected from

South Amherst, MA in 1975 by Phillip Ives (vid. (Rose 1984)), and then cultured in the lab using two-week discrete generations. These ancestral IV flies were subsequently used in February 1980 to create five "O" (old) replicate lines (Rose 1984). The IV flies were also used to found five additional "B" (baseline) populations in February, 1980, populations which have since been cultured using the same protocol as the IV line from which they were derived (see Figure 4.2).

Culture regimes: Over subsequent years, additional treatments were derived from the O populations using three distinct culture regimes, of which two are studied here: "A," "and "C" (see Figure 4.3). ***A*** *culture regime*: the five ACO and five AO populations spend their first 9 days of life in 8-dram glass vials, and at day 10 are transferred to a Plexiglass "cage" in which they are given fresh food and allowed to oviposit for 24 hours. ***C*** *culture regime:* the five CO and five nCO populations develop in vials for 14 days prior to being transferred to Plexiglass cages. C flies are then given 48 hours to oviposit before eggs are collected on day 28. All populations are supplied with food made from cooked bananas, barley malt, yeast, corn syrup, and agar. The populations that spend time in cages are also supplied with live yeast on the medium surface prior to egg laying.

Test-Cross Experimental Design

Spoke crossing system: Two sets of within-treatment and between-treatment experiments were performed using the ten A-type and ten C-type populations in a "spoke" fashion. Under C-type regime conditions, we performed the following series of crosses, for i values from 1 to 5: $CO_i \times CO_j$; $CO_i \times nCO_i$; $CO_i \times ACO_i$; $CO_i \times ACO_j$, where i is not equal to j. Under A-type regime conditions, we performed the following series of crosses: $ACO_i \times ACO_j$; $ACO_i \times AO_i$; $ACO_i \times CO_i$; $ACO_i \times CO_j$, where i is not equal to j. [See Figure 4.1.] For each cross between

stock populations, three types of flies were assayed: ancestor ("**a"**), cohabiting ("**c"**), and hybrid ("**F_1"**). A second-generation ("**F_2**") was also used, but only for the development duration rate assay. Ancestor flies were obtained from crosses between males and females from a single ancestral population (e.g. all flies sampled from ACO_1). Cohabiting flies have females from one ancestral population living with males of another ancestral population (e.g. ACO_1 females cohabiting with CO_2 males). Hybrid flies are the F_1 offspring of a co-habiting population cross (e.g. when we write $ACO_1 \times CO_2$, we refer to hybrids between population ACO_1 and CO_2).

Rearing and sampling of assay cohorts: D. melanogaster cultures were initiated (day 0) in 25×95 mm vials containing 20 ml of banana/agar/yeast media at a density of 70 eggs per natal vial for each population test-cross.

In order to maintain regime-specific conditions throughout the experiment, special natal vials were created. These natal vials were made of two components: a 23×25mm cap and a 25×95 tube. The cap containing fly medium was inserted into the tube to create a vial of standard dimensions. After reaching the appropriate development stage, larvae would then climb the walls of the tube to pupate.

Virgin adults were collected using light CO_2 anesthesia as they eclosed from their pupal cases on day 8 for the ACO populations and day 10 for CO populations. Flies were sexed, crossed, and then placed into holding vials that consisted of a cap from the natal vials inserted into a clean tube. Population test-crosses were made by combining 25 virgin flies of each gender in the females' natal-capped holding vial, ten vials per population-cross, with 500 flies total for each such cross.

Adults were allowed to mate and freely interact in the females' natal vials until the normal culture day: day 10 for the A-type regime. The C-type regime flies were transferred into

cages at day 14, and then eggs were collected from those cages on days 26 to 28, from egg. These experimental procedures closely mimicked the normal culture regime experienced by the ancestor populations (see Figure. 4.3). Thus the assays performed in this study provide a reasonable estimate of fitness under the culture conditions that each type of fly had experienced for hundreds of generations.

Overview of our analytical strategy

The analysis of our data is based on a specific scenario for the resumption of contact between flies from different lab populations, conceived as a model system for incipient reproductive isolation between long-allopatric populations. As this scenario is somewhat complicated, we have provided both a schematic, shown in Figure 4.4, and the following verbal summary.

If two populations have long been divided by a major geographic barrier, the likelihood that a single migrant will undermine their reproductive isolation is remote. What we have in mind instead is the following scenario.

Step One. A propagule from one geographical area migrates to the other geographical area, where this propagule consists of enough individuals so that it does not suffer from notable inbreeding depression. Note, however, that this initial migration is not assumed to immediately lead to hybridization. Thus the initial analytical question is how well this propagule group can survive under the selective conditions imposed in the geographical area to which it has newly migrated.

Step Two. Over some part of its range in its new geographical area, the migrant propagule group cohabits with the endemic population that has long undergone adaptation to that geographical area. During this phase of the process, matings may occur between endemic and

propagule individuals specifically in the zone of cohabitation. At this point, *prezygotic* components of reproductive isolation come into play.

Step Three. As a result of hybridization events, some part of the geographical area inhabited by migrant and endemic individuals constitutes a hybrid zone, in which hybrids and individuals with uncrossed parental genomes constitute a mixed population. At this point, the relative fitness of hybrid progeny compared to individuals with uncrossed parental genomes plays an important evolutionary role. This relative fitness difference can then be assayed with respect to *postzygotic* components of reproductive isolation.

Reproductive Isolation Assays

Our assays tested both prezygotic and postzygotic reproductive isolation characters, comparing hybrids to uncrossed individuals for each assay. The prezygotic characters tested were (i) survival to the time of mating and (ii) mate choice. The postzygotic characters tested were (i) fecundity and (ii) development. Uncrossed and hybrid individuals are tested simultaneously by deriving the hybrids from the previous parental generation.

Life-history assays covered the entire range of ages during which any of the tested populations are maintained in our lab, from day 0 (from egg) to day 28 (from egg). This time period includes the longest duration that any adult fly is allowed to live in our present stock system, which no longer includes the O populations of Rose (1984). Selection-regime "focal" fitness was calculated from data collected specifically during the reproductive window of each treatment's generation cycle (as shown in Figure 4.3).

Mate choice assay: Two mate-choice tests were used: (1) a female from an endemic population was given the choice to mate with one of two suitors: a male from her own population

and a male from a migrant population; and (2) same procedure as test (1), but with a migrant female as the choosing female instead of an endemic female. Each male was given colored yeast paste to ingest for identification purposes, with rotating combinations of colors among types of male. The flies were given two hours in which to mate. A successful mating event was scored when a male mounted a female for thirty seconds or more. Mate choice assays were conducted at 24 hours from eclosion using virgin flies. If females did not mate at all within the two hours, the experimental trial was discarded. Males were classified as (i) mated or not mated, (ii) marked or not marked. Sixty choice-assays were performed for each type of test.

Adult survival assay: Flies from holding vials were transferred to population cages at each treatment's normal day of transfer out of their rearing vials: day 9 for ACO flies and day 14 for CO flies. Population cages were surveyed for dead flies before food plates were replaced each day. The dead flies were removed, then sexed, and their number recorded. Each cage housed 500 flies.

Net fecundity assay: Each assay cage contained a single Petri dish of food medium. Almost all of the eggs were laid in or on that Petri dish. During the adult survival assay, the Petri dish was removed from the cage daily. The removed Petri dish was rinsed with bleach solution in order to collect all the laid eggs onto a membrane placed within a Buchner funnel. The membrane was then placed on a flatbed scanner and photographed. The number of eggs laid was counted from this photographic image using ImageJ software. Net fecundity at a particular age is normally rendered as k_x ($k_x = l_x\, m_x$, where l_x is probability of survival to age x and m_x is fecundity at age x).

Development assay: Under A-type and C-type regime conditions, 20 vials of ~70 eggs were collected from the population cages on the normal culture day, days 10 and 28 respectively.

For the ACO and CO treatments a fraction of the number of eggs that were laid during focal fecundity were used for development. The number of eggs that developed into adults was recorded every 4 hours from day 8 to day 14, the last day on which adults are collected from vials. The number of adults that eclosed during the development duration assay was added to the focal fecundity total.

Due to differences in development rates, C-type flies cannot successfully establish themselves in an A-type environment. A-type flies develop in vials for nine days before adult flies are transferred into cages to begin laying eggs. C-type flies begin to eclose from pupae towards the end of day ten, and thus are reproductively isolated from ACO flies under A-type regime conditions. To estimate differences in fecundity between CO and ACO flies *under an ad hoc regime that is less stringent*, we measured k_x using 11-day old flies. In addition, eggs laid during day eleven for CO, and cohabiting populations (CO males × ACO females; ACO males × CO females) were sampled to measure developmental success. Developmental success was analyzed by first aligning the data to account for differences in time between egg collections.

Reproductive Isolation Values

We quantified reproductive isolation between populations using a composite measure derived from the test-cross data. Our component measures of Reproductive Isolation Value (RIV) specify the strength of reproductive isolation inferred from each test-cross assay. This component index of reproductive isolation was calculated using the method proposed by Coyne and Orr (1989; 1997)

$$RIV_n = 1 - \frac{competitor}{maternal\,ancestor} \qquad (1)$$

where the subscript *n* refers to the specific character under study (e.g. mate choice). All these

component indices of isolation reflect statistically significant differences between "competitor" (e.g. hybrid) and ancestral individuals in each test cross. RIV estimates are expected to vary between negative and positive values, where one is complete reproductive isolation. Scenarios in which hybridization is favored, as a consequence of disassortative mating or hybrid vigor, result in negative reproductive isolation values.

To calculate a composite measure of reproductive isolation, we used the method proposed by Ramsey et al. (2003): where the "life-history stage contribution" ("*LHC"*) of a component of reproductive isolation value (*RIV*) at stage *n* in the life history is calculated in the following manner:

$$LHC_1 = RIV_1, \tag{2}$$

$$LHC_2 = RIV_2\left(1 - LHC_1\right), \text{ and} \tag{3}$$

$$LHC_3 = RIV_3\left[1 - (LHC_1 + LHC_2)\right]. \tag{4}$$

Generally:
$$LHC_n = RIV_n\left(1 - \sum_{i=1}^{n-1} LHC_i\right). \tag{5}$$

In this parameterization, a particular component reproductive barrier is taken to eliminate gene flow that has not already been prevented by previous components of reproductive isolation. To calculate total reproductive isolation in our study, seven sequential life-history stage components were used: 1) focal fecundity of the ancestor populations; 2) developmental success of ancestral progeny; 3) mate choice between ancestor-population flies; 4) focal fecundity of the cohabiting flies; 5) developmental success of F_1 hybrids; 6) focal fecundity of the F_1 hybrids; and 7) developmental success of F_2 hybrids. For *m* components of reproductive isolation, total reproductive isolation (*T*) is

$$T = \sum_{i=1}^{m} LHC_i \,. \qquad (6)$$

As *T* reaches one (1), reproductive isolation becomes complete.

F_{ST} estimates: F_{ST} estimates were calculated at every single nucleotide polymorphism ("SNP") across the major chromosome arms to estimate genetic differentiation among within-treatment populations, as well as genetic differentiation between populations from different selection treatments. To do this, SNPs were first called across the 30 populations of the Rose stock system used in this dissertation. SNPs were discarded if coverage in any of the populations was less than 20X or greater than 500X. We also required a minimum minor allele frequency of 2% across all 30 populations. Based on these criteria, ~1.13 million SNPs were identified across the major chromosome arms. A SNP table with major and minor allele counts for each SNP in each population was then generated.

SNP frequencies were taken directly from these counts and F_{ST} estimates were obtained using the formula: $F_{ST} = (H_T - H_S)/H_T$ where H_T is heterozygosity calculated from the overall allele frequencies, and H_S is the average observed heterozygosity in each replicate population (Hedrick 2009). Estimates were made at every SNP across the major chromosome arms. This calculation was done between replicate ancestral test pairs.

Data Analysis

Mate-choice data: The counts of males in each of the possible cells from this experiment were analyzed using a chi-square test. Marking was necessary to distinguish between males from different population crosses. However, since it may impact the females' preference, we controlled for this by rotating colors amongst types of males. Therefore, we were able to test whether mating status is independent of marking status in each experiment.

Survival and Fecundity data: We tested for differences in survival and fecundity due to population-cross status over relative and absolute ages using a linear mixed effects model. The observations consisted of fecundity at a particular age (*t*) but within a small age interval. These age intervals were chosen to span the ages such that all comparison populations still had live flies. Within each interval, survival or fecundity rates were modeled by a straight line and allowing population type (*j*= 1 (**a** 1), , 2 (**a** 2), , 3 (**c** 1), 4 (**c** 2), 5 ($\mathbf{F_1}$ 1), 6 ($\mathbf{F_1}$ 2)) to affect the intercept of that line but not the slope. However, slopes were allowed to vary between intervals. As with the other analyses, cages (*i*=1,…,12) were assumed to contribute random variation to these measures. With this notation, the survival (or fecundity) at age-*t*, interval-*k*, selection regime-*j* and population-*i*, is y_{ijkt} and is described by,

$$y_{ijkt} = \alpha + \beta_k + \delta_j\gamma_j + (\omega + \pi_k\delta_k)t + \delta_k\delta_j\mu_{jk} + c_i + \varepsilon_{ijkt} \qquad (7)$$

where δ_s =0 if *s*=1 and 1 otherwise and c_i, and ε_{ijkt} are independent standard normal random variables with variance σ_c^2 and σ_ε^2 respectively. The effects of selection on the intercept are assessed by considering the magnitude and variance of both γ_j and μ_{jk}.

Development data: Successful adult emergence prior to culture day was analyzed with the same analysis as fecundity using a linear mixed effects model. The observations consisted of developmental success percentage at a particular age (*t*) but within a small time interval. These time intervals were chosen to span the development window prior to culture day. Within each interval, developmental success percentage were modeled by a straight line and allowing population type (*j*= 1 (**a** 1), , 2 (**a** 2), , 3 (**c** 1), 4 (**c** 2), 5 ($\mathbf{F_1}$ 1), 6 ($\mathbf{F_1}$ 2)) to affect the intercept of that line but not the slope. However, slopes were allowed to vary between intervals. As with the other analyses, population cages (*i*=1,…,12) were assumed to contribute random variation to

these measures. With this notation, the developmental success at age-*t*, interval-*k*, selection regime-*j* and population-*i*, is y_{ijkt} and is described by,

$$y_{ijkt} = \alpha + \beta_k + \delta_j\gamma_j + (\omega + \pi_k\delta_k)t + \delta_k\delta_j\mu_{jk} + c_i + \varepsilon_{ijkt} \qquad (7)$$

where δ_s =0 if *s*=1 and 1 otherwise and c_i, and ε_{ijkt} are independent standard normal random variables with variance σ_c^2 and σ_ε^2 respectively. The effects of selection on the intercept are assessed by considering the magnitude and variance of both γ_j and μ_{jk}.

Results

In this study, we evaluated three qualitatively different kinds of characters: developmental success, mate choice, and net fertility at age x, the last also known as k_x. These three kinds of characters were in turn evaluated for as many as sixty different types of fly, with each combination of fly-type and assay-type involving up to 1,200 individual flies. This is a massive body of data. For ease of reader comprehension, we have relegated the detailed analyses of all these particular combinations of fly-type and assay to Appendix A. Reported in this Results section are the life-history stage components (LHC) of reproductive isolation for each assay, averaged across all five replicates. LHC calculations are presented in two ways: (1) with all RI-values regardless of statistical significance; and (2) with only statistically significant RI-values. LHC averages using all RI-values, method (1), are shown in order to present all the "noise" obscuring the state of potential incipient reproductive isolation across every assay. The normal practice in the field (e.g. Ramsey et al. 2003) is to take non-significant signals of reproductive isolation as zero, method (2). Interestingly there are only minor differences between the ultimately calculated levels for overall RI obtained using the two methods. For that

reason, it is not of much scientific significance that the RI values calculated using method (2) were used to draw general conclusions.

At first, the data for this Chapter were acquired in the same manner as the data collection for Chapters 2 and 3, with absolute comparisons of the life-history components of reproductive isolation. Cursory analysis revealed complete reproductive isolation when C-type flies invade A-type populations under A-type conditions; simply put, the C's were eliminated immediately. Stated another way, we have even stronger evidence in support of ecological speciation mechanisms, and against null speciation mechanisms, in this Chapter, when the data are collected and analyzed using the same methods as in other Chapters. In addition, these results preclude any additional "Interaction" effect of additional generations in allopatry in this particular case, where C flies fail to establish themselves under A conditions. To get around this last problem for the case of C flies invading A populations, we adjusted our experimental design to offer possible routes by which C flies might invade A populations, by somewhat relaxing the A -type selection regime. However, with this more accommodating procedure, we were able to evaluate whether there are interactions between evolutionary duration and selective differentiation, with respect to reproductive isolation.

ACO invading ACO

We begin by looking at the question of how readily ACO-type flies establish themselves in an A-type environment, in the face of implicit competition with endemic ACO flies. This is parametrically defined by the "Ancestor k_x" reproductive isolation value, which measures the difference in fecundity between migrant and endemic populations under endemic conditions. This is the first component of reproductive isolation between populations from the ACO

treatment. The average k_x net difference among ACO populations, as shown in the first line of part B of Table 4.1, is -0.084. A negative k_x value indicates that on average more eggs are produced by the immigrant ACO populations than by the endemic populations, which is hardly a signal of reproductive isolation. Next we consider how readily eggs from immigrant ACO's develop under A-type conditions. The average net difference in developmental success between migrant and endemic ACO cohorts, as given in the second line of part B of Table 4.1, is 0.024. From these two results, it is evident that immigrants from another long-allopatric ACO population can easily establish a hybrid zone shared with endemic ACO populations.

In such hybrid zones, individuals from migrant and endemic populations will come in direct contact and possibly mate. The average mate-choice preference between migrant and endemic ACO males is zero, meaning that ACO females do not significantly prefer males from their population of origin, as shown in line three of part B of Table 4.1. Thus hybrid matings will occur in the hybrid zone, and mixed populations will arise with members from migrant and endemic populations cohabiting with their hybrids.

The next task is to determine whether there is a difference in fecundity between cohorts that are entirely made up of flies from different ancestral populations (e.g. all ACO_1 males cohabiting with all ACO_2 females, as an entire experimental cohort) and cohorts made up entirely of males and females that share the same population of origin. As shown in line four of part B of Table 4.1, there are no statistically significant differences in k_x values between hybridizing flies and endemics. Eggs laid by cohabiting populations will thus produce F_1 hybrids in the hybrid zone. Line five of part B of Table 4.1 indicates that there are some small differences between F_1 individuals and endemics with respect to developmental success, with an average net difference of 0.042, the largest such effect to be found among ACO comparisons.

But we find no statistically significant differences in the net fecundity of F_1 hybrids compared to those of endemic populations, as shown in line six of part B of Table 4.1. Thus these hybrid zones are likely to feature F_2 hybrids. The developmental success of such F_2 hybrids was compared to the endemic populations, producing an average net difference of 0.040, as shown in line seven of part B of Table 4.1. Combining these values together, we get a summed estimate of total reproductive isolation between ACO populations of 0.022 ± .11 (standard error). In other words, there is no statistically significant reproductive isolation among ACO populations, as already found in the experiments of Chapter 2. [Please note, however, that the data analyzed here are entirely new. So this is a statistically independent confirmation of the findings of Chapter 2.]

AO_i invading ACO_i with recent common ancestor O_i

AO flies have only recently evolved under A-type regime conditions, though AO_i flies share a common ancestor O_i ancestor with ACO_i flies. We begin by looking at the "Ancestor k_x" reproductive isolation values between A-type populations. The average k_x net difference between AO_i and ACO_i populations, as shown in the first line of part B of Table 4.2, is zero. This indicates no difficulty for AO propagule groups invading habitat that contains ACO individuals. Next we consider how readily eggs from immigrant AO_i's develop under A-type conditions. The average net difference in developmental success between migrant and endemic populations, as given in the second line of part B of Table 4.2, is -0.015. This negative value indicates that immigrant AO_i develop on average faster than do endemic ACO_i populations facing invasion. From these two results, it is evident that immigrants from AO_i populations can easily establish a hybrid zone shared with endemic ACO_i populations.

In such hybrid zones, individuals from migrant and endemic populations will come in direct contact and possibly mate. The average mate-choice preference between migrant AO_i and endemic ACO_i males is zero, meaning that both A-type females do not significantly prefer males from their population of origin, as shown in line three of part B of Table 4.2. Thus hybrid matings will occur in the hybrid zone, and mixed populations will arise with members from migrant and endemic populations cohabiting with their hybrids.

The next question is to determine whether there is a difference in fecundity between cohorts that are entirely made up of flies from different ancestral populations (e.g. all AO_1 males cohabiting with all ACO_1 females, as an entire experimental cohort) and cohorts made up entirely of males and females that share the same population of origin. As shown in line four of part B of Table 4.2, the average net reproductive isolation value of k_x values between hybridizing flies and endemics is 0.017. Eggs laid by cohabiting populations will thus produce F_1 hybrids in the hybrid zone. Line five of part B of Table 4.2 indicates that there are some small differences between F_1 individuals and endemics with respect to developmental success, with an average net difference of -0.058. We also find statistically significant differences in the net fecundity of F_1 hybrids compared to those of endemic populations, with an average net difference of -0.131 as shown in line six of part B of Table 4.2. A negative value suggests the F_2 hybrids produce on average more eggs than the endemic populations. The developmental success of such F_2 hybrids was compared to the endemic populations, producing an average net difference of 0.104, as shown in line seven of part B of Table 4.2. Combining these values together, we get a summed estimate of total reproductive isolation between AO and ACO populations of -0.083 ± .23 (standard error). In other words, there is, at most, statistically insignificant hybrid vigor between

AO_i and ACO_i populations. There is no evidence for any type of reproductive isolation between them, despite their having undergone about 1,100 generations of independent evolution.

CO_i invading ACO_i with recent common ancestor O_i

We begin by looking at the question of how readily C-type flies establish themselves in an A-type environment. Due to differences in development rates, C-type flies cannot successfully establish themselves in an A-type environment, as already mentioned at the outset of this Results section. A-type flies develop in vials for nine days before adult flies are transferred into cages to begin laying eggs. C-type flies begin to eclose from pupae towards the end of day ten, and thus are reproductively isolated from ACO flies under A-type regime conditions. To estimate differences in fecundity between CO and ACO flies *under an ad hoc regime that is less stringent*, we measured k_x using 11-day old flies. The average k_x net difference among ACO_i and CO_i populations with this protocol, as shown in the first line of part B-section (i) of Table 4.3, is zero. As mentioned above, the differences in developmental success are absolute between these two types of flies with an average reproductive isolation value of 1.000, under the stringent assay conditions that we have used up to this point.

To assess any possible "Interaction" effect in fostering reproductive isolation, we have further relaxed our invasion scenario in order to test for mate-choice preferences using males at the age of 12 days from egg, though using test ACO females at an age of 10 days. In the kind of ecologically less stringent hybrid zones that we have experimentally emulated, individuals from migrant and endemic populations will come in direct contact and possibly mate. The average mate-choice preference between migrant CO_i and endemic ACO_i males by ACO_i females is zero. Using 12-day-old CO_i females choosing between 12-day-old CO_i males and 10-day-old ACO_i

males, the average mate-choice preference between migrant CO_i and ACO_i males by CO_i females was 0.597. We average these two mate-preference values to calculate the life-history contribution of mate-choice to reproductive isolation, producing an average reproductive isolation value for mate-choice of 0.299, as shown in line one of part B-section (ii) of Table 4.3. Thus hybrid matings will occur in the hybrid zone, but more often between ACO females and CO males than the reciprocal cross.

The next question is to determine whether there is a difference in fecundity between cohorts that are entirely made up of flies from different ancestral populations (e.g. all ACO_1 males cohabiting with all CO_1 females, as an entire experimental cohort) and cohorts made up entirely of males and females that share the same population of origin. As shown in line two of part B-section (ii) of Table 4.3, there are no statistically significant differences in k_x values between hybridizing flies and endemics. Eggs laid by cohabiting populations will thus produce F_1 hybrids in the hybrid zone. Line three of part B-section (ii) of Table 4.3 indicates that there are large differences between F_1 individuals and endemics with respect to developmental success, with an average net difference of 0.492, the largest such effect to be found in part B-section (ii) of Table 4.3. But we find little statistically significant differences in the net fecundity of F_1 hybrids compared to those of endemic populations, as shown in line four of part B-section (ii) of Table 4.3. Thus these hybrid zones are likely to feature F_2 hybrids. The developmental success of such F_2 hybrids was compared to the endemic populations, producing an average net difference of 0.174, as shown in line five of part B-section (ii) of Table 4.3. Combining these values together, we get a summed estimate of total reproductive isolation between CO_i and ACO_i populations of 0.970 ± .01 (standard error). Even with the exclusion of the effects of developmental differences between ancestral populations during initial CO invasion, there is still

statistically significant reproductive isolation between CO_i and ACO_i populations that share a common O_i ancestor.

CO_i invading ACO_j without a recent common ancestor

In the preceding analysis, the CO_i and ACO_i populations that were tested for reproductive isolation share a recent common O_i ancestor. Here we test CO_i and ACO_j populations that do not share a recent common ancestor. Once again, we have complete reproductive isolation between ACO_i and CO_j populations, when CO flies invade ACO populations. But again we used mitigating ecological circumstances, and measured the difference in fecundity between migrant and endemic flies by comparing fecundity between 11-day old ACO_i and CO_j flies. The average k_x net difference among ACO and CO populations, as shown in the first line of part B-section (i) of Table 4.4, is zero. As mentioned above the differences in developmental success are absolute with an average reproductive isolation value of 1.000.

We relaxed our invasion scenario and begin our analysis with mate-choice. In such hybrid zones, individuals from migrant and endemic populations will come in direct contact and possibly mate. The average mate-choice preference between migrant CO_i and endemic ACO_j males by ACO_j females is 0.144. The average mate-choice preference between migrant CO_i and ACO_j males by CO_i females is 0.524. We average these two values to calculate the life-history contribution of mate-choice to reproductive isolation. The average net reproductive isolation value for mate-choice is 0.335, as shown in line one of part B-section (ii) of Table 4.4. Thus hybrid matings will occur in the hybrid zone, but arise more readily from matings of ACO females and CO males than the reciprocal alternative.

The next question is to determine whether there is a difference in fecundity between cohorts that are entirely made up of flies from different ancestral populations (e.g. all ACO_2 males cohabiting with all CO_1 females, as an entire experimental cohort) and cohorts made up entirely of males and females that share the same population of origin. As shown in line two of part B-section (ii) of Table 4.4, there are no statistically significant differences in k_x values between hybridizing flies and endemics. Eggs laid by cohabiting populations will thus produce F_1 hybrids in the hybrid zone. Line three of part B-section (ii) of Table 4.4 indicates that there are large differences between F_1 individuals and endemics with respect to developmental success, with an average net difference of 0.491, the largest such effect to be found in part B-section (ii) of Table 4.4. But we find little statistically significant differences in the net fecundity of F_1 hybrids compared to those of endemic populations, as shown in line four of part B-section (ii) of Table 4.4. Thus these hybrid zones are likely to feature F_2 hybrids. The developmental success of such F_2 hybrids was compared to the endemic populations, producing an average net difference of 0.134, as shown in line five of part B-section (ii) of Table 4.4. Combining these values together, we get a summed estimated of total reproductive isolation between CO_i and ACO_j populations of 0.982 ± .01 (standard error). Once again there is statistically significant reproductive isolation between CO_i and ACO_j populations, even when we impose less stringent conditions on the invading CO flies.

CO_i invading CO_j

We begin by looking at the question of how readily CO-type flies establish themselves in an C-type environment, in the face of implicit competition with endemic CO flies. This is parametrically defined by the "Ancestor k_x" reproductive isolation value, which measures the

difference in fecundity between migrant and endemic populations. This is the first component of reproductive isolation between populations from the CO treatment. The average k_x net difference among CO populations, as shown in the first line of part B of Table 4.5, is -0.061. A negative k_x value indicates on average more eggs are produced by migrant CO flies than endemic CO flies. Note that under C-type conditions, with 14 days allowed for development, all flies develop in sufficient time to be included in the next generation and therefore a developmental success assay was not used in this reproductive isolation analysis.

Given the likelihood of hybrid zones, individuals from migrant and endemic populations will come in direct contact and sometimes mate. The average mate-choice preference between migrant and endemic CO males is zero, meaning that CO females do not significantly prefer males from their population of origin, as shown in line two of part B of Table 4.5. Thus hybrid matings will occur in the hybrid zone, and mixed populations will arise with members from migrant and endemic populations cohabiting with their hybrids.

The next question is to determine whether there is a difference in fecundity between cohorts that are entirely made up of flies from different ancestral populations (e.g. all CO_1 males cohabiting with all CO_2 females, as an entire experimental cohort) and cohorts made up entirely of males and females that share the same population of origin. As shown in line three of part B of Table 4.5, there are no statistically significant differences in k_x values between hybridizing flies and endemics. We find no statistically significant differences in the net fecundity of F_1 hybrids compared to those of endemic populations, as shown in line four of part B of Table 4.5. Thus these hybrid zones are likely to feature F_2 hybrids. Combining these values together, we get a summed estimate of total reproductive isolation between CO populations of -0.160 ± .16

(standard error). In other words, there is no statistically significant overall reproductive isolation among CO populations in these experiments, as already found in the data of Chapter 2.

nCO_i invading CO_i with recent common O_i ancestor

nCO_i flies have recently evolved under C-type regime conditions from ancestral O_i and share a recent common O_i ancestor with CO_i flies. We begin by looking at the "Ancestor k_x" reproductive isolation values between A-type populations. The average k_x net difference between nCO_i and CO_i populations, as shown in the first line of part B of Table 4.6, is zero. Immigrants from nCO_i populations can easily establish a hybrid zone shared with endemic CO_i populations.

In such hybrid zones, individuals from migrant and endemic populations will come in direct contact and possibly mate. The average mate-choice preference between migrant nCO_i and endemic CO_i males by CO_i females is -0.132. The average mate-choice preference between migrant nCO_i and endemic CO_i males by nCO_i females is zero. We use both values to calculate the life-history contribution of mate-choice to reproductive isolation. The average net reproductive isolation value between females across all replicates for mate-choice is -0.066, as shown in line two of part B of Table 4.6. A negative value for mate-choice favors hybridization. Thus hybrid matings will occur in the hybrid zone, but proceed more readily between CO_i females and nCO_i males than the reciprocal alternative.

The next question is to determine whether there is a difference in fecundity between cohorts that are entirely made up of flies from different ancestral populations (e.g. all nCO_1 males cohabiting with all CO_1 females, as an entire experimental cohort) and cohorts made up entirely of males and females that share the same population of origin. As shown in line three of part B of Table 4.6, the average reproductive isolation value of k_x values between hybridizing

flies and endemics is zero. Eggs laid by cohabiting populations will thus produce F_1 hybrids in the hybrid zone. We also find no statistically significant differences in the net fecundity of F_1 hybrids compared to those of endemic populations, with an average difference of zero as shown in line four of part B of Table 4.6. Combining these values together, we get a summed estimate of total reproductive isolation between nCO_i and CO_i populations of -0.066 ± .14 (standard error). In other words, there is statistically insignificant of hybrid vigor between nCO and CO populations, and no signal of incipient reproductive isolation.

ACO_i invading CO_i with recent common O_i ancestor

We begin by looking at the question of how readily A-type flies establish themselves in a C-type environment. The reproductive isolation found between A-type and C-type flies caused by developmental differences in the A-type environment do not occur in the C-type environment. All fly types, including A-type flies, develop well within the 14 days imposed by the C-type environment. We begin by reporting the difference in fecundity between migrant and endemic populations. The average k_x net difference between populations, as shown in the first line of part B of Table 4.7, is 0.725. Next we measure mate preference with the average value between migrant ACO_i and endemic CO_i males by ACO_i females is zero. However, the average mate-choice preference between migrant ACO_i and CO_i males by CO_i females is 0.584. We use both values to calculate the life-history contribution of mate-choice to reproductive isolation. The average net reproductive isolation value for mate-choice is 0.079, as shown in line two of part B of Table 4.7. Thus hybrid matings will occur in the hybrid zone, but proceed more readily between ACO females and CO males than the reciprocal cross.

The next question is to determine whether there is a difference in fecundity between cohorts that are entirely made up of flies from different ancestral populations (e.g. all ACO_1 males cohabiting with all CO_1 females, as an entire experimental cohort) and cohorts made up entirely of males and females that share the same population of origin. As shown in line three of part B of Table 4.7, there are statistically significant differences in k_x values between hybridizing flies and endemics. But we find no statistically significant differences in the net fecundity of F_1 hybrids compared to those of endemic populations, as shown in line four of part B of Table 4.7. Thus these hybrid zones are likely to feature F_2 hybrids. Combining the life-history component values of reproductive isolation together, we get a summed estimated of total reproductive isolation between CO and invading ACO populations of 0.918 ± .02 (standard error). Thus there is clear evidence of reproductive isolation having arisen from the different selection histories of these two kinds of population.

ACO_i invading CO_j without a recent common ancestor

In the preceding analysis, the ACO_i and CO_i populations tested share a recent common O_i ancestor. Here we test ACO_i and CO_j populations that do not share a recent common ancestor. We begin by reporting the difference in fecundity between migrant and endemic populations. The average k_x net difference between populations, as shown in the first line of part B of Table 4.8, is 0.779. Next we measure mate preference with the average value between migrant ACO_i and endemic CO_j males by ACO_i females is zero. However, the average mate-choice preference between migrant ACO_i and CO_j males by CO_j females is 0.557. We use both values to calculate the life-history contribution of mate-choice to reproductive isolation. The average net reproductive isolation value for mate-choice is 0.063, as shown in line two of part B of Table

4.8. Thus hybrid matings will occur in the hybrid zone, but proceed more readily between ACO females and CO males than the reciprocal cross.

The next question is to determine whether there is a difference in fecundity between cohorts that are entirely made up of flies from different ancestral populations (e.g. all ACO_2 males cohabiting with all CO_1 females, as an entire experimental cohort) and cohorts made up entirely of males and females that share the same population of origin. As shown in line three of part B of Table 4.8, there are statistically significant differences in k_x values between hybridizing flies and endemics. But we find no statistically significant differences in the net fecundity of F_1 hybrids compared to those of endemic populations, as shown in line four of part B of Table 4.8. Thus these hybrid zones are likely to feature F_2 hybrids. Combining the life-history component values of reproductive isolation together, we get a summed estimated of total reproductive isolation between CO_i and invading ACO_j populations of 0.936 ± .01 (standard error).

Overall Combined Analysis of the Effects of Selection and Generation Number

The results indicate two general trends: 1) selection is relatively a greater force than evolutionary time in initiating reproductive isolation; and 2) the signal generated by selection is strong enough to be detected fairly undiminished using RI-values regardless of statistical significance. Whether there is an interaction of selection and time in fostering reproductive isolation will require an additional analysis.

A linear mixed effect model was used to analyze differences between endemic and migrant populations in net fecundity, endemic male mating success, and developmental success. For each assay in our invasion scenario, three hypotheses were tested regarding the initiation of reproductive isolation: 1) is there an effect of generation time; 2) is there an effect of selection

regime; and 3) is there an interaction between generation time and selection regime. The results are shown in Table 4.9 and they confirm the conclusions drawn from the RIV analyses regarding the relative importance between evolutionary time and selection. In addition, we find no evidence of the interaction between selection regime and evolutionary time in initiating reproductive isolation.

F_{st} mean estimates between ancestral population test-pairs.

Genome-wide sequencing studies have been performed with all 20 of the populations studied here (Graves et al., in prep.). Here we report the estimates of F_{ST} values for the pairwise differentiation between the replicate populations of the two selection regimes we have used in this study (See Table 4.10). Sampling of flies for genome-wide sequencing occurred a year prior to the phenotypic assays being conducted. The number of generations between genome wide sequencing and the phenotypic assays was 36 for A-type flies and 13 for C-type flies. Given what little time has passed between genomic and phenotypic assays, we believe inferences regarding the relationship between genomic differentiation and insipient reproductive isolation can still be made.

We begin by looking at F_{ST} values for populations that share a selection regime and evolutionary history. ACO populations have evolved in parallel in allopatry for 1,800 generations and have an overall F_{ST} value averaged across all five replicates of $0.037 \pm .02$. The CO populations have evolved in C-type regime conditions for 750 generations and have an overall average F_{ST} value of $0.025 \pm .01$.

Next we look at population pairs that are evolving under the same selection regime, but do not share identical evolutionary histories. The ACO and AO populations are both evolving in

an A-type environment and share a common ancestor in the O populations. The ACO populations were derived from the CO populations that were in turn derived from the O populations. The AO populations were derived directly from the O populations. The number of generations between test pairs is 1,160 with an overall average F_{ST} value of 0.045 ± .02. The O populations are also the common ancestor of the C-type flies, with the nCO populations being recently derived. There are 480 generations between the CO and nCO populations with an average F_{ST} value of 0.022 ± .01.

We now turn our attention to comparisons between A-type and C-type flies. A-type flies have a 10-day generation cycle and have a faster development rate than the C-type populations. The C-type populations have a 28-day generation cycle and live considerably longer than A-type flies. F_{ST} values were calculated between A-type and C-type test pairs with 815 generations, as well as 1,560 generations between them. In both cases, the overall average F_{ST} value between A-type and C-type flies is 0.063 ± .02.

Overall, there are larger differences in F_{ST} values between ACO_i-CO_i and ACO_i-CO_j population pairs than there are between population pairs of the same type (i.e., CO_i-CO_j; CO_i-nCO_i). There does not seem to be an effect of time in increasing differentiation when comparing F_{ST} values of population pairs between scenarios of the same experiment (See Table 4.10). There does seem to be an effect of time when comparing F_{ST} values among A-type population pairs (ACO_i-ACO_j; ACO_i-AO_i) to the F_{ST} values among C-type population pairs (CO_i-CO_j; CO_i-nCO_i). It is possible that these differences in F_{ST} values may be a reflection of the evolutionary pressures unique to specific selection regimes and not necessarily the effect of evolutionary time.

Discussion

Overview of the Present Results

Surveying the repeated patterns of the results obtained for the individual invasion scenarios, as well as considering the overall integrated analysis of the last subsection of the Results, clearly indicates that the predominant factor in determining incipient reproductive isolation is ecological selection. We find little evidence to support *any* null evolutionary genetic scenario for the evolution of reproductive barrier. Nor do we find any material evidence suggesting an interaction between duration of allopatry, denominated by number of generations, and ecological speciation. All these results qualitatively support the salience of ecological speciation theory only.

Comparison with Previous Results from this Dissertation

The results of this Chapter 4 are entirely in keeping with the results of Chapters 2 and 3. Firstly, this Chapter includes almost exactly equivalent analyses of crosses among ACO populations and among CO populations, with qualitatively similar results as in Chapter 2. In addition, this Chapter 4 contains similar crosses among A-type populations and among C-type populations with different evolutionary histories, respectively. That is to say, in this Chapter 4 we consider cases in which A-type populations with very different evolutionary histories are crossed with each other, and likewise there are crosses among C-type populations with very different evolutionary histories. But again, no significant signals of reproductive isolation were found, among all types of crosses that share recent selection histories. Evidently, recent functional convergence produced by selection obliterated any impact of past ecological

differentiation on reproductive isolation. There is no evidence in this Chapter either for the action of null mechanisms of speciation.

Similarly to Chapter 3, we find a consistent and strong signal of reproductive isolation between populations that have recently adapted to different environments. In this Chapter 4, we used different population-crosses than were used in Chapter 3, in the present case C-type populations crossed with A-type populations as opposed to the crosses of C-type and B-type populations in Chapter 3. Nonetheless the same kind of findings were obtained. In fact, under strict A-type conditions, C-type populations are entirely unable to initially invade. We were only able to study the success of subsequent steps in our invasion scenario by relaxing the stringency of the A-type selection regime. Thus the findings of this Chapter 4 are even more corroborative of the ecological speciation theory than the findings of Chapter 3.

Comparisons with other studies of speciation

Let's begin by asking whether there are any other studies in the published literature on speciation that significantly address whether the number of generations in allopatry interacts with ecological speciation. To this point in our review of the literature, we have found no such studies.

Thus we are once again left with the general question of the relative merits of ecological speciation theory versus the broad array of alternative hypotheses in the published literature on the evolution of reproductive isolation. We will defer that discussion to Chapter 5.

Conclusion

This Chapter 4 provides a still more stringent set of tests for both null mechanisms of speciation, as well as ecological mechanisms of speciation. It also tests for any signal of interaction between these two qualitatively distinct speciation mechanisms. In keeping with our earlier findings in this dissertation, we find no evidence for the action of null mechanisms of speciation, despite the maintenance of strictly parallel selection histories over as many as 900 generations. Also in keeping with our earlier findings, we find strong evidence for the action of ecological speciation. With respect to the selection and culture regimes imposed in our laboratory, ACO and CO populations are effectively reproductively isolated from each other already. Finally, we find no statistical evidence for any contribution of generation number to the effects of ecological differentiation in the establishment of reproductive isolation.

Acknowledgements

We thank the hundreds of undergraduate researchers in the lab of M.R.R. who were involved in conducting these experiments. This work was supported by a DDIG Grant NSF-DEB-1311644 awarded to L.G.C and M.R.R and a GAANN Fellowship awarded to L.G.C. from the Department of Education.

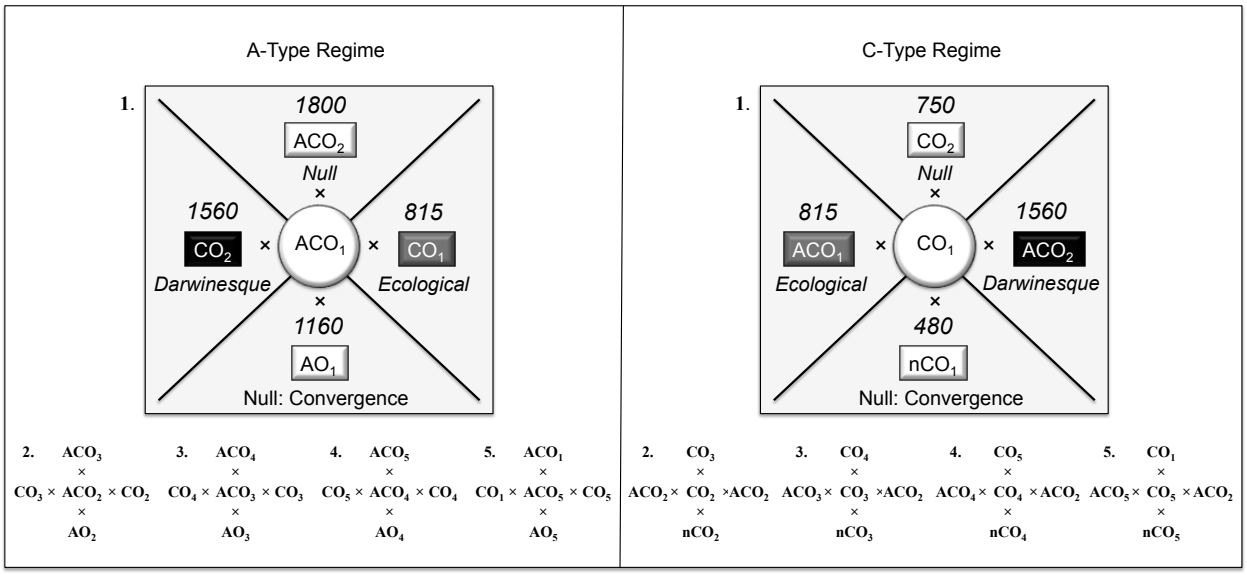

Figure 4.1. Population design crosses that give signals for the combined action of null and ecological speciation mechanisms in the evolution of our laboratory populations. Listed are the numbers of generations of evolutionary divergence. Five replicate tests were conducted under both A-type and C-type environments.

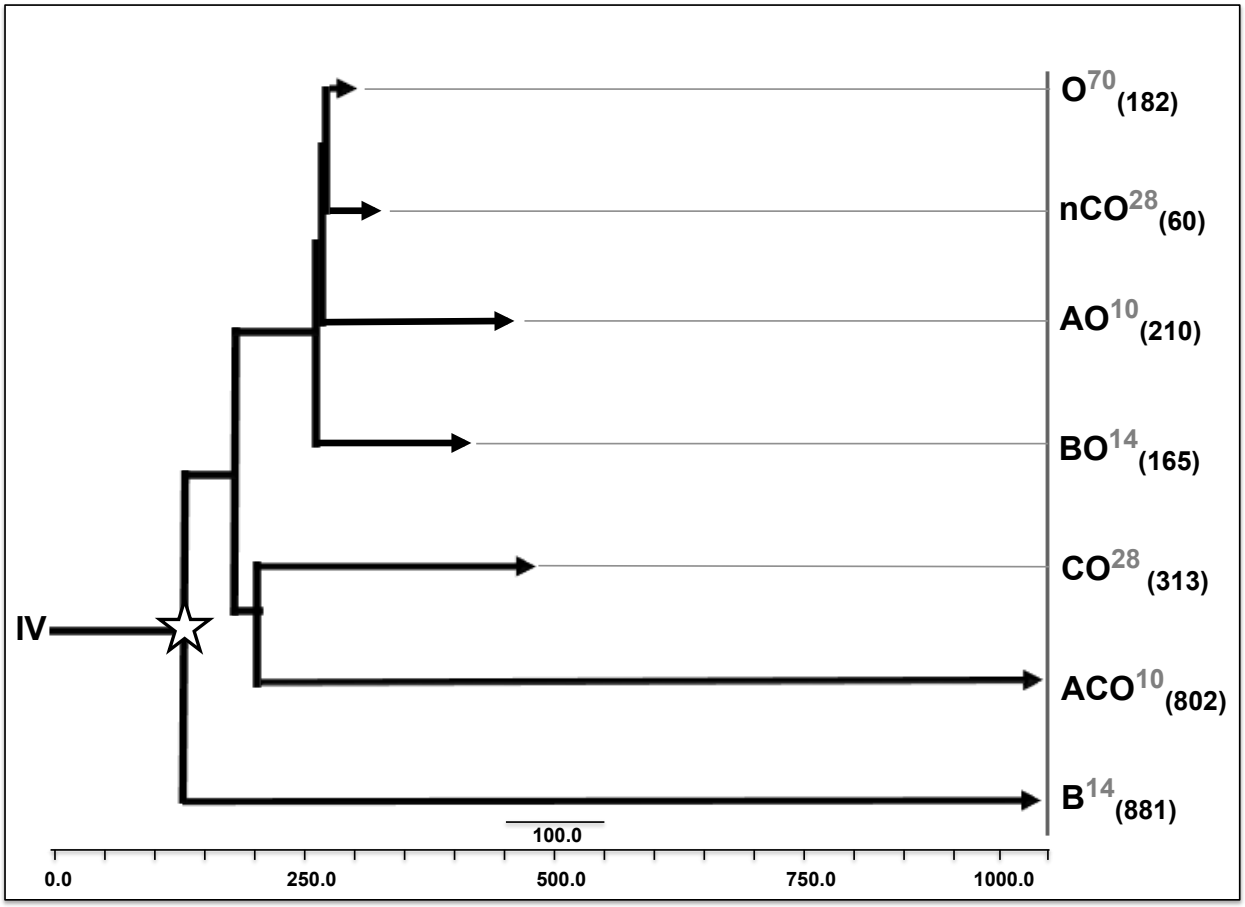

Figure 4.2. Collection of allopatric Drosophila laboratory populations derived from a single outbred population ("IV") in early1980 (star). Each selection regime was imposed on five populations. The X-axis gives the number of generations evolving under laboratory conditions. The Y-axis shifts indicate changes in selection regimes, with the life-cycle length of each selection regime indicated by the superscript, and the number generations evolving under distal selection regime indicated by the subscript.

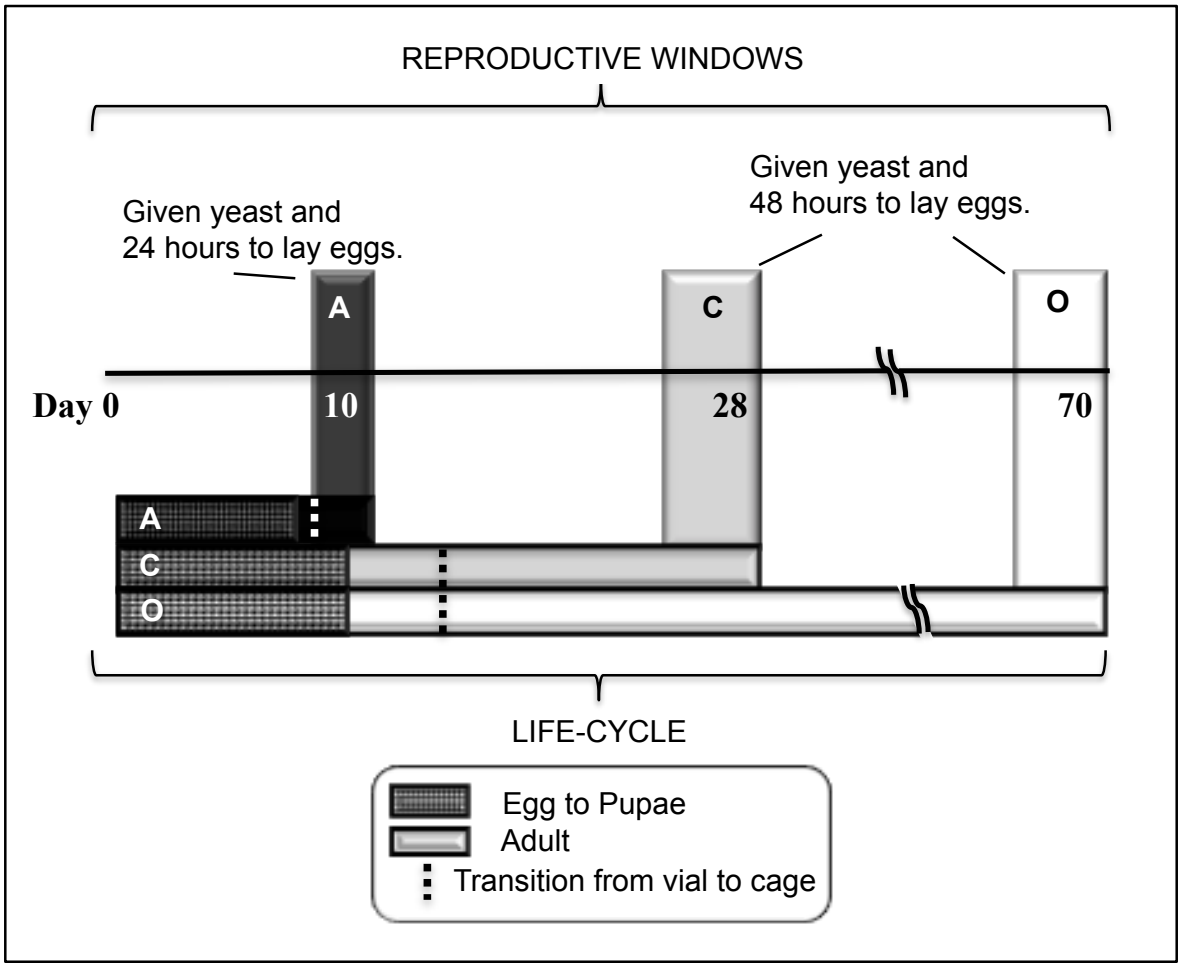

Figure 4.3. Distinct selection regimes imposed on five-fold replicated groups of outbred populations. The primary difference between selection regimes is the time interval (reproductive window) when eggs are collected to establish the next generation. Only A-type and C-type populations were used in this study.

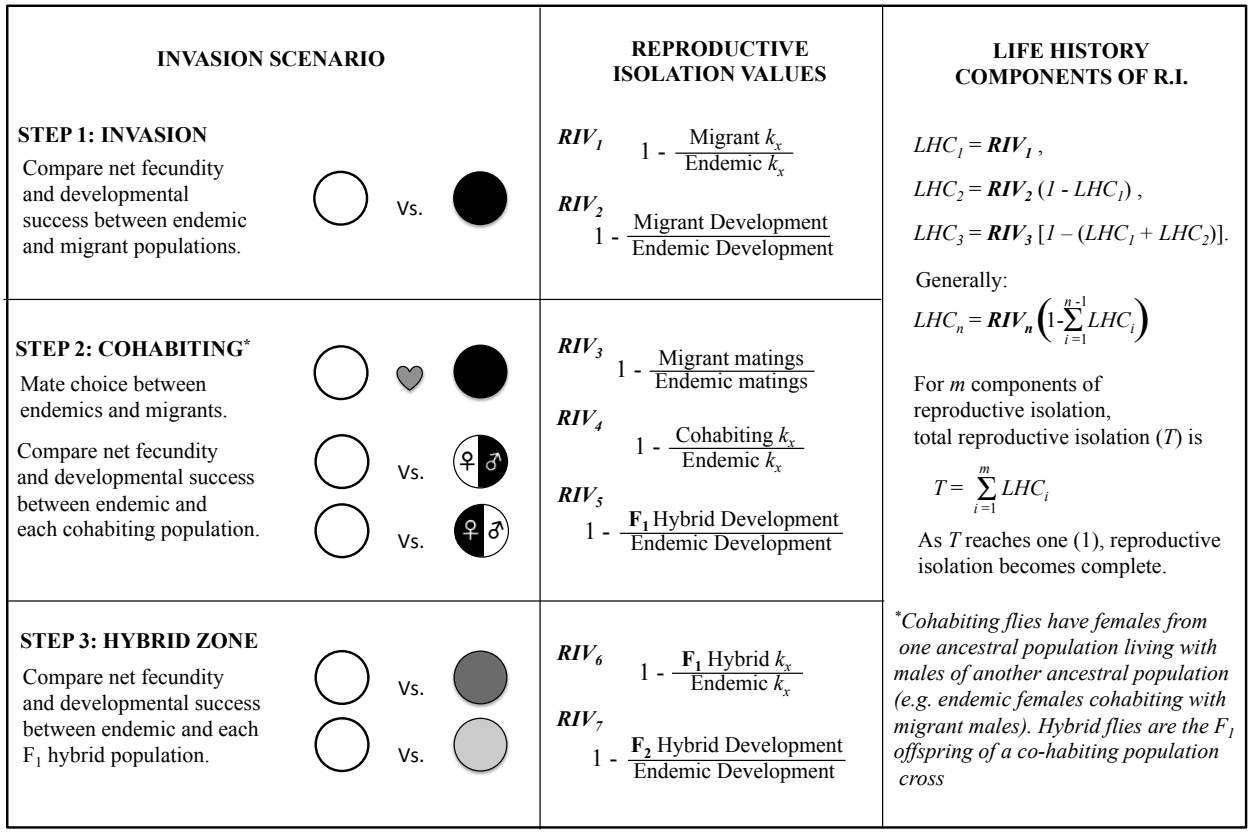

Figure 4.4. Schematic of a secondary contact scenario conceived as a model system for incipient reproductive isolation between allopatric populations. A composite measure of reproductive isolation was calculated using sequential life-history stage components.

Table 4.1. Summary of reproductive isolation for ACO test-crosses under the A-type culture-regime environment. Isolation components vary from zero (no barrier) to one (complete isolation). Negative component values indicate life-history stages at which hybridization is favored. Part A lists life history components calculated with RI-values regardless of statistical significance. Part B lists life history components that are calculated with only RI-values that are statistically significant.

ACO_i invades ACO_j ($i \neq j$): Life History Components of Reproductive Isolation

Average R.I.	R.I. Barrier	Replicates					LHC Ave.
		1	2	3	4	5	
A)	Ancestor k_x	0.113	0.181	-0.421	-0.151	0.132	-0.029
	Ancestor Dev.	0.106	0.083	-0.021	0.085	-0.017	0.047
All R.I. Values	Mate-choice	-0.123	-0.018	0.080	-0.180	0.143	-0.020
0.018 ± .21	Cohabiting k_x	0.049	-0.100	-0.047	0.052	-0.083	-0.026
	F_1 Hybrid Dev.	-0.052	0.129	-0.214	0.047	0.027	-0.013
	F_1 Hybrid k_x	0.113	0.091	-0.055	-0.098	-0.072	-0.004
	F_2 Hybrid Dev.	0.076	0.097	-0.098	0.221	0.019	0.063
	Total	0.280	0.463	-0.775	-0.025	0.149	0.018
B)	Ancestor k_x	0.000	0.000	-0.421	0.000	0.000	-0.084
	Ancestor Dev.	0.119	0.000	0.000	0.000	0.000	0.024
Stat. Sig. R.I.V.	Mate-choice	0.000	0.000	0.000	0.000	0.000	0.000
0.022 ± .11	Cohabiting k_x	0.000	0.000	0.000	0.000	0.000	0.000
	F_1 Hybrid Dev.	0.000	0.127	0.000	0.000	0.084	0.042
	F_1 Hybrid k_x	0.000	0.000	0.000	0.000	0.000	0.000
	F_2 Hybrid Dev.	0.050	0.000	0.000	0.152	0.000	0.040
	Total	0.169	0.127	-0.421	0.152	0.084	0.022

Table 4.2. Summary of reproductive isolation for ACO and AO test-crosses under the A-type culture-regime environment. Isolation components vary from zero (no barrier) to one (complete isolation). Negative component values indicate life-history stages at which hybridization is favored. Part A lists life history components calculated with RI-values regardless of statistical significance. Part B lists life history components that are calculated with only RI-values that are statistically significant.

AO_j invades ACO_j: Life History Components of Reproductive Isolation

Average R.I.	R.I. Barrier	Spoke Replicates					LHC Ave.
		1	2	3	4	5	
A)	Ancestor k_x	0.269	-0.262	-0.194	-0.160	-0.106	-0.091
	Ancestor Dev.	-0.036	0.049	-0.275	-0.218	0.295	-0.037
All R.I. Values	Mate-choice	0.084	-0.202	-0.071	0.040	0.126	-0.005
-0.341 ± .40	Cohabiting k_x	0.161	-0.919	-0.249	0.165	-0.026	-0.174
	F_1 Hybrid Dev.	0.073	0.020	-0.462	0.032	-0.046	-0.077
	F_1 Hybrid k_x	-0.017	0.320	0.000	-0.070	-0.478	-0.049
	F_2 Hybrid Dev.	0.223	0.101	-0.289	0.346	0.069	0.090
	Total	0.758	-0.893	-1.541	0.134	-0.165	-0.341
B)	Ancestor k_x	0.000	0.000	0.000	0.000	0.000	0.000
	Ancestor Dev.	0.000	0.000	-0.115	-0.094	0.134	-0.015
Stat. Sig. R.I.V.	Mate-choice	0.000	0.000	0.000	0.000	0.000	0.000
-0.083 ± .23	Cohabiting k_x	0.194	-0.391	0.000	0.281	0.000	0.017
	F_1 Hybrid Dev.	0.000	0.000	-0.288	0.000	0.000	-0.058
	F_1 Hybrid k_x	0.000	0.000	0.000	-0.103	-0.552	-0.131
	F_2 Hybrid Dev.	0.383	0.043	-0.163	0.257	0.000	0.104
	Total	0.578	-0.348	-0.566	0.341	-0.418	-0.083

Table 4.3. Summary of reproductive isolation for ACO and CO test-crosses, that share a recent common ancestor, under the A-type culture-regime environment. Isolation components vary from zero (no barrier) to one (complete isolation). Negative component values indicate life-history stages at which hybridization is favored. Part A lists life history components calculated with RI-values regardless of statistical significance. Part B lists life history components that are calculated with only RI-values that are statistically significant.

CO_j invading ACO_j: Life History Components of Reproductive Isolation

Average R.I.	R.I. Barrier	Replicates					LHC Ave.
		1	2	3	4	5	
A) All R.I. Values							
	Ancestor k_x	-0.200	-0.146	-0.051	-0.148	-0.178	-0.145
i. With Propagule	Ancestor Dev.	1.200	1.146	1.051	1.148	1.178	1.145
1.000 ± .00	Total	1.000	1.000	1.000	1.000	1.000	1.000
	Mate-choice	0.375	0.311	0.244	0.310	0.474	0.343
ii. No Propagule	Cohabiting k_x	0.096	0.078	0.110	0.073	0.054	0.082
0.986 ± .01	F_1 Hybrid Dev.	0.337	0.326	0.544	0.550	0.410	0.434
	F_1 Hybrid k_x	-0.048	0.086	0.039	0.000	0.024	0.020
	F_2 Hybrid Dev.	0.223	0.161	0.053	0.063	0.037	0.107
	Total	0.982	0.962	0.990	0.995	0.998	0.986
B) Stat. Sig. R.I.V.							
	Ancestor k_x	0.000	0.000	0.000	0.000	0.000	0.000
i. With Propagule	Ancestor Dev.	1.000	1.000	1.000	1.000	1.000	1.000
1.000 ± .00	Total	1.000	1.000	1.000	1.000	1.000	1.000
	Mate-choice	0.271	0.273	0.261	0.268	0.420	0.299
ii. No Propagule	Cohabiting k_x	0.000	0.000	0.000	0.000	0.000	0.000
0.970 ± .01	F_1 Hybrid Dev.	0.404	0.361	0.592	0.628	0.478	0.492
	F_1 Hybrid k_x	-0.119	0.099	0.000	0.000	0.046	0.005
	F_2 Hybrid Dev.	0.396	0.208	0.117	0.094	0.054	0.174
	Total	0.952	0.94	0.969	0.99	0.997	0.970

Table 4.4. Summary of reproductive isolation for ACO and CO test-crosses, that do not share a recent common ancestor, under the A-type culture-regime environment. Isolation components vary from zero (no barrier) to one (complete isolation). Negative component values indicate life-history stages at which hybridization is favored. Part A lists life history components calculated with RI-values regardless of statistical significance. Part B lists life history components that are calculated with only RI-values that are statistically significant.

CO*i* invading ACO*j* ($i \neq j$): Life History Components of Reproductive Isolation

Average R.I.	R.I. Barrier	Replicates					LHC Ave.
		1	2	3	4	5	
A) All R.I. Values							
	Ancestor k_x	-0.040	-0.140	0.013	-0.104	-0.133	-0.081
i. With Propagule	Ancestor Dev.	1.040	1.140	0.987	1.104	1.133	1.081
1.000 ± .00	Total	1.000	1.000	1.000	1.000	1.000	1.000
	Mate-choice	0.273	0.617	0.296	0.348	0.189	0.345
ii. No Propagule	Cohabiting k_x	-0.310	0.000	0.106	0.045	0.008	-0.030
0.984 ± .01	F_1 Hybrid Dev.	0.609	0.247	0.455	0.582	0.655	0.510
	F_1 Hybrid k_x	-0.013	0.031	0.067	0.001	0.048	0.027
	F_2 Hybrid Dev.	0.393	0.090	0.067	0.021	0.094	0.133
	Total	0.953	0.985	0.992	0.998	0.996	0.984
B) Stat. Sig. R.I.V.							
	Ancestor k_x	0.000	0.000	0.000	0.000	0.000	0.000
i. With Propagule	Ancestor Dev.	1.000	1.000	1.000	1.000	1.000	1.000
1.000 ± .00	Total	1.000	1.000	1.000	1.000	1.000	1.000
	Mate-choice	0.297	0.617	0.23	0.258	0.276	0.335
ii. No Propagule	Cohabiting kx	0.000	0.000	0.000	0.000	0.000	0.000
0.982 ± .01	F_1 Hybrid Dev.	0.345	0.250	0.578	0.704	0.580	0.491
	F_1 Hybrid kx	0.000	0.023	0.035	0.000	0.048	0.021
	F_2 Hybrid Dev.	0.310	0.094	0.138	0.036	0.091	0.134
	Total	0.952	0.984	0.981	0.997	0.995	0.982

Table 4.5. Summary of reproductive isolation for CO test-crosses under the C-type culture-regime environment. Isolation components vary from zero (no barrier) to one (complete isolation). Negative component values indicate life-history stages at which hybridization is favored. Part A lists life history components calculated with RI-values regardless of statistical significance. Part B lists life history components that are calculated with only RI-values that are statistically significant.

CO_i invading CO_j $(i \neq j)$: Life History Components of Reproductive Isolation

Average R.I.	R.I. Barrier	Replicates					LHC Ave.
		1	2	3	4	5	
A)	Ancestor k_x	0.125	0.023	0.222	0.257	-0.305	0.065
All R.I. Values	Mate-choice	0.128	0.214	-0.113	0.031	-0.342	-0.017
-0.110 ± .30	Cohabiting k_x	-0.186	-0.020	-0.027	0.050	0.039	-0.029
	F_1 Hybrid k_x	-0.261	-0.083	0.254	0.055	-0.612	-0.130
	Total	-0.194	0.134	0.335	0.392	-1.220	-0.110
B)	Ancestor k_x	0.000	0.000	0.000	0.000	-0.305	-0.061
Stat. Sig. R.I.V.	Mate-choice	0.000	0.000	0.000	0.000	0.000	0.000
-0.160 ± .16	Cohabiting k_x	0.000	0.000	0.000	0.000	0.000	0.000
	F_1 Hybrid k_x	0.000	0.000	0.000	0.000	-0.497	-0.099
	Total	0.000	0.000	0.000	0.000	-0.801	-0.160

Table 4.6. Summary of reproductive isolation for CO and nCO test-crosses under the C-type culture-regime environment. Isolation components vary from zero (no barrier) to one (complete isolation). Negative component values indicate life-history stages at which hybridization is favored. Part A lists life history components calculated with RI-values regardless of statistical significance. Part B lists life history components that are calculated with only RI-values that are statistically significant.

nCO_j invading CO_j: Life History Components of Reproductive Isolation

Average R.I.	R.I. Barrier	Spoke Replicates					LHC Ave.
		1	2	3	4	5	
A)	Ancestor k_x	0.082	-0.304	0.134	0.274	-0.284	-0.020
All R.I. Values	Mate-choice	-0.017	-0.824	0.069	-0.265	0.416	-0.124
-0.281 ± .36	Cohabiting k_x	-0.153	-0.073	0.024	-0.035	-0.277	-0.103
	F_1 Hybrid k_x	-0.198	-0.415	0.134	0.335	-0.026	-0.034
	Total	-0.286	-1.616	0.361	0.309	-0.171	-0.281
B)	Ancestor k_x	0.000	0.000	0.000	0.000	0.000	0.000
Stat. Sig. R.I.V.	Mate-choice	0.000	-0.588	0.000	0.000	0.257	-0.066
-0.066 ± .14	Cohabiting k_x	0.000	0.000	0.000	0.000	0.000	0.000
	F_1 Hybrid k_x	0.000	0.000	0.000	0.000	0.000	0.000
	Total	0.000	-0.588	0.000	0.000	0.257	-0.066

Table 4.7. Summary of reproductive isolation for CO and ACO test-crosses, that share a recent common ancestor, under the C-type culture-regime environment. Isolation components vary from zero (no barrier) to one (complete isolation). Negative component values indicate life-history stages at which hybridization is favored. Part A lists life history components calculated with RI-values regardless of statistical significance. Part B lists life history components that are calculated with only RI-values that are statistically significant.

ACO_j invading CO_j: Life History Components of Reproductive Isolation

Average R.I.	R.I. Barrier	CO Spoke Replicates					LHC Ave.
		1	2	3	4	5	
A)	Ancestor k_x	0.786	0.758	0.602	0.824	0.653	0.725
All R.I. Values	Mate-choice	0.065	0.089	0.114	0.075	0.021	0.073
0.865 ± .03	Cohabiting k_x	0.043	0.052	0.105	0.027	0.119	0.069
	F_1 Hybrid k_x	-0.015	0.023	0.025	-0.009	-0.033	-0.002
	Total	0.879	0.922	0.846	0.917	0.760	0.865
B)	Ancestor k_x	0.786	0.758	0.602	0.824	0.653	0.725
Stat. Sig. R.I.V.	Mate-choice	0.070	0.080	0.103	0.051	0.089	0.079
0.918 ± .02	Cohabiting k_x	0.080	0.110	0.162	0.070	0.151	0.114
	F_1 Hybrid k_x	0.000	0.000	0.000	0.000	0.000	0.000
	Total	0.935	0.948	0.866	0.945	0.894	0.918

Table 4.8. Summary of reproductive isolation for CO and ACO test-crosses, that do not share a recent common ancestor, under the C-type culture-regime environment. Isolation components vary from zero (no barrier) to one (complete isolation). Negative component values indicate life-history stages at which hybridization is favored. Part A lists life history components calculated with RI-values regardless of statistical significance. Part B lists life history components that are calculated with only RI-values that are statistically significant.

ACO_i invading CO_j ($j \neq i$): Life History Components of Reproductive Isolation

Average R.I.	R.I. Barrier	Replicates					LHC Ave.
		1	2	3	4	5	
A)	Ancestor k_x	0.715	0.857	0.748	0.833	0.744	0.779
All R.I. Values	Mate-choice	0.121	0.059	0.083	0.054	0.063	0.076
0.906 ± .03	Cohabiting k_x	0.069	0.050	0.061	0.035	0.061	0.055
	F_1 Hybrid k_x	-0.003	0.014	0.001	0.005	-0.043	-0.005
	Total	0.903	0.980	0.893	0.928	0.826	0.906
B)	Ancestor k_x	0.715	0.857	0.748	0.833	0.744	0.779
Stat. Sig. R.I.V.	Mate-choice	0.087	0.033	0.065	0.043	0.088	0.063
0.936 ± .01	Cohabiting k_x	0.119	0.070	0.108	0.070	0.098	0.093
	F_1 Hybrid k_x	0.000	0.001	0.000	0.000	0.000	0.000
	Total	0.921	0.960	0.921	0.946	0.929	0.936

Table 4.9. Summary of the data analysis of the effects of selection and generation number on initiating reproductive isolation.

A-type Experiment			C-type Experiment		
Invasion Stage	Effect	*p*-value	Invasion Stage	Effect	*p*-value
Ancestor k_x	Generation	0.491	Ancestor k_x	Generation	0.700
	Selection Regime	0.815		Selection Regime	0.014*
	Interaction	0.878		Interaction	0.518
Ancestor Dev.	Generation	1.000			
	Selection Regime	0.000*			
	Interaction	0.193			
Mate-choice	Generation	0.310	Mate-choice	Generation	0.779
	Selection Regime	0.096		Selection Regime	0.022*
	Interaction	0.053		Interaction	0.681
Cohabiting k_x	Generation	0.052	Cohabiting k_x	Generation	0.516
	Selection Regime	0.048*		Selection Regime	0.363
	Interaction	0.079		Interaction	0.966
F_1 Hybrid Dev.	Generation	0.932			
	Selection Regime	0.000*			
	Interaction	0.595			
F_1 Hybrid k_x	Generation	0.524	F_1 Hybrid k_x	Generation	0.496
	Selection Regime	0.094		Selection Regime	0.416
	Interaction	0.514		Interaction	0.300
F_2 Hybrid Dev.	Generation	0.835			
	Selection Regime	0.000*			
	Interaction	0.200			

Table 4.10. Mean F_{ST} estimates across the major chromosome arms between cohort pairs based on SNP frequencies. Sampling of flies for genome-wide sequencing occurred a year prior to phenotypic assays being conducted.

Scenario	Populations used in A-type Experiment			Populations used in C-type Experiment		
	Populations	Gen.	Mean F_{st}	Populations	Gen.	Mean F_{st}
Null	ACO_1-ACO_2	1800	0.032	CO_1-CO_2	750	0.022
	ACO_2-ACO_3	1800	0.043	CO_2-CO_3	750	0.021
	ACO_3-ACO_4	1800	0.039	CO_3-CO_4	750	0.024
	ACO_4-ACO_5	1800	0.036	CO_4-CO_5	750	0.030
	ACO_5-ACO_1	1800	0.035	CO_5-CO_1	750	0.028
Null: "Convergence"	ACO_1-AO_1	1160	0.038	CO_1-nCO_1	480	0.015
	ACO_2-AO_2	1160	0.044	CO_2-nCO_2	480	0.023
	ACO_3-AO_3	1160	0.048	CO_3-nCO_3	480	0.022
	ACO_4-AO_4	1160	0.047	CO_4-nCO_4	480	0.022
	ACO_5-AO_5	1160	0.049	CO_5-nCO_5	480	0.028
Ecological	ACO_1-CO_1	815	0.061	CO_1-ACO_1	815	0.061
	ACO_2-CO_2	815	0.062	CO_2-ACO_2	815	0.062
	ACO_3-CO_3	815	0.066	CO_3-ACO_3	815	0.066
	ACO_4-CO_4	815	0.052	CO_4-ACO_4	815	0.052
	ACO_5-CO_5	815	0.073	CO_5-ACO_5	815	0.073
Interaction	ACO_1-CO_2	1560	0.066	CO_1-ACO_2	1560	0.062
	ACO_2-CO_3	1560	0.061	CO_2-ACO_3	1560	0.066
	ACO_3-CO_4	1560	0.069	CO_3-ACO_4	1560	0.049
	ACO_4-CO_5	1560	0.055	CO_4-ACO_5	1560	0.069
	ACO_5-CO_1	1560	0.067	CO_5-ACO_1	1560	0.067

CHAPTER 5

Conclusion: Speciation in Light of Experimental Evolution

Abstract

Since Darwin published the *Origin of Species*, biologists have developed and refined concrete hypotheses for the evolutionary mechanics that lead to the creation of new species by branching off from extant species. That is, there has been a 150 year proliferation of theories of speciation. A variety of publications has recently attempted to interpret data from naturally occurring populations of the various theories that have been proposed. But, with a few singular exceptions, evolutionary biologists have not endeavored to test their theories of speciation using well-replicated experiments. This dissertation is an initial attempt to use experimental evolution to clear away theories of speciation that are not viable. In particular, we present extensive experimental data which suggest that speciation is not likely to occur in the absence of patterns of natural selection that differ between allopatric populations. In terms of the terminology used in this dissertation, we consider "null" theories of speciation substantively falsified here. In contrast, ecological speciation theories are supported by our findings. There are conceivable scenarios in which null mechanisms of speciation might operate, which we discuss. We also address criticisms of our experimental system, with respect to its relevance as a tool for testing speciation theories. Finally, we discuss whether the relative success of ecological speciation theories is another instance of the prepotency of natural selection within evolutionary genetics, a particularly Darwinian view that has been under sustained attack over the last 50 years.

Introduction

As reviewed in Chapter 1, there is a wide range of theories of speciation that have been proposed ever since Charles Darwin effectively created evolutionary biology in 1859 with his epochal *Origin of Species*. While Darwin was a great equivocator, particularly when he didn't have clear evidence to reason from, many evolutionary biologists have since come forward with detailed and cogent theories of speciation, couched particularly in terms of genetic mechanisms since Theodosius Dobzhansky's 1937 *Genetics and the Origin of Species.*

Like some others (vid. Schluter 2000, 2001; Rundle and Nosil 2005; Schluter 2009, Nosil et al. 2009) in Chapter 1 we organized this wide range of speciation hypotheses into two broad categories: ecological speciation theories and "null" speciation theories. One way to think of this dichotomy is that the null speciation theories are the other part of the Venn diagram, once ecological speciation theories are grouped together in one sprawling subset of the Venn diagram. There is some historical cogency to this division, in that Darwin developed the primordia of ecological speciation theory, while subsequent writers about speciation before the Modern Synthesis, such as Hugo De Vries (Gayon 1998, pp. 255-60), were evidently interested in developing speciation theories that did *not* rest on a foundation of natural selection. Since the Modern Synthesis of 1918-1945 (Mayr 1982), subsequent work on the problem of speciation has of course greatly increased the sophistication and empirical content of both kinds of speciation theory (vid. Coyne and Orr 2004; Schulter 2009). Nonetheless, this long-standing contrast between speciation theories that depend on adaptation driven by natural selection and those that do not has been sustained.

But more importantly for the purpose of this dissertation, the ecological/null dichotomy leads fairly directly to experimental tests based on the standing diversity among the *Drosophila*

melanogaster populations that have evolved in the Rose laboratory since 1980 (vid. Rose et al. 2004; also Burke et al. in prep., Graves et al. in prep.). As the experiments of Chapters 2, 3, and 4 of this dissertation instantiate, the Rose laboratory's system of populations is set up in a manner which is ideal for testing the relative merits of ecological versus null theories of speciation, even though there was no such intent before 2009.

A Popperian context for the present research

Up until the latter part of the 20th Century, biologists were often faced with the problem of phenomena for which they had no well-developed theoretical explanation. The range of such unexplained phenomena was notably broad: aging, sex, the origin of life, biological altruism, and so on. Since 1960, however, there has been a proliferation of cogent, often formally developed, theories for most of these longstanding mysteries of life. Speciation has been no exception to this rule, as conveyed in Chapter 1.

For scientists influenced by the works of the epistemologist Karl R. Popper (e.g. Popper 1959 – Logic of Scientific Discovery), this theoretical proliferation calls for pruning. That is to say, while it is fun for theoreticians and those who read their works to learn of yet another theory for the evolution of sex, for example, the proliferation of such theories is not entirely satisfying for those scientists who believe that scientific progress depends on the winnowing of a diversity of theories. On the other hand, there are few things harder in science than performing strong inference experiments that clearly demolish one theory while supporting another (vid. Platt 1966). And evolutionary biology is no exception to this general rule.

Some of those who work in the field of speciation research have been quite aware of the challenges that this particular field has faced with respect to the design and execution of strong-inference experiments (e.g. Schluter and Conte 2009). Schluter and Conte (2009) thus laid down a set of desiderata for such experiments, as follows:

> We focus on progress in understanding ecological speciation between adjacent marine and stream-resident threespine stickleback populations. The marine species or ecotype is the ancestral form to all freshwater populations.
>
> The stream stickleback populations exhibit rampant parallel evolution of morphological traits. Virtually everywhere it occurs, the stream ecotype is smaller in size, less streamlined in shape, and has reduced armor compared with the marine species. Size and other differences between stickleback populations have been shown in laboratory common-garden studies to be substantially heritable.
>
> The strongest evidence for ecological speciation in this group comes from a test of ‘‘parallel speciation’’ demonstrating repeated evolution of reproductive isolation between populations across a similar environmental gradient (quoted in Schluter and Conte 2009).

When we learned of this “call to arms” from Schluter and others (e.g. Via 2009), we realized that the system of populations in the Rose laboratory more or less was ideal for testing the relative merits of null versus ecological speciation theories. This was of particular interest to us, because we had long before delineated criteria by which molecular genetic theories of speciation might be empirically evaluated (Rose and Doolittle 1983). Thus the scientific challenge, as posed quite well by Schluter and Conte (2009), became performing strong-inference tests that would decide the relative merits of ecological and null theories of speciation within the well-defined and extensively replicated system of populations that we had already developed.

This opportunity was further enhanced by a large-scale genome-wide sequencing project that was conducted with 30 of the Rose laboratory populations in 2012 (Graves et al., in prep.).

That project gave us genome-wide surveys of single nucleotide polymorphism (SNP) data, data which enabled us to address with some certainty issues of genetic differentiation and the like among the populations of our experimental system.

We feel that it is reasonable to claim that the experimental findings reported in Chapters 2, 3, and 4 constitute a significant achievement with respect to the winnowing of theories of speciation. In this respect, we would like to point out that we began this project with no particular favorite between ecological and null theories of speciation. Indeed, Rose and Doolittle (1983) argued for the plausibility of a particular null mechanism of speciation, speciation due to the differential proliferation of transposable elements among allopatric populations. Regardless of our initial hypotheses, however, we found no material evidence in support of null mechanisms of speciation. We found these results, as summarized in Chapter 2, despite having maintained five-fold replicated populations for hundreds of generations in three separate cases.

In striking, and again unanticipated, contrast, Chapters 3 and 4 provide an abundance of evidence suggesting that laboratory natural selection readily generates functional differentiation among divergently selected populations sufficient to foster reproductive isolation. Under some protocols, such as the invasion of C-type populations under A-type (very early reproduction) conditions, reproductive isolation arising from ecological mechanisms of divergent selection is essentially complete.

Thus, as Popperians, we find ourselves having produced a strong-inference kind of result: falsification of null theories of speciation combined with corroboration of ecological theories of speciation in a well-defined and extensively replicated laboratory radiation. From this, admittedly not universal, Popperian perspective, the present body of work seems scientifically useful. We have, in effect, cleared some of the theoretical underbrush obstructing the scientific

appraisal of the causes of cladogenic speciation. Naturally, this claim calls for appropriate qualification and delimitation.

Alternative Scenarios for Null Speciation?

Any laboratory evolution project suffers from material limitations on its generality. In the present project, we used *D. melanogaster* populations with moderate N_e, around 1,000 (Mueller et al. 2013) and evolutionary durations not much greater than 1,000 generations. How relevant are these conditions for the falsification of null theories of speciation?

Firstly, given the abundance of standing genetic variation involved in these populations (Burke et al. 2010; Graves et al., in prep.; see also Chapter 2, this dissertation), it is not likely that this experimental system fails as a model for testing null speciation theories because of a lack of genetic variation. Nor is it the case that this genetic variation is solely neutral, as many sites across the genome respond to directional selection on life history in this system (Burke et al. 2010; Graves et al., in prep.). Populations with still larger effective population sizes will feature more standing genetic variation (cf. Mackay et al. 2012), and thus still more rapid responses to functional selection during allopatry.

Secondly, we have managed to sustain relatively stable, parallel, culture conditions across groups of populations for almost 1,000 generations in some cases. Would still more generations of such sustained parallel selection provide a better case for testing null models of speciation? Our view is that such stringently parallel selection is extremely unlikely to be sustained in nature even for as long as we have in the Rose laboratory. With respect to stable parallel patterns of

selection among allopatric populations, we find the idea that *all* relevant patterns of selection will feature such parallelism over hundreds of generations implausible.

Thirdly, we would suggest that the best redoubt for null models of speciation is to focus on the evolution of small N_e populations, particularly in well-defined systems of Mendelian experimental evolution. With small N_e, experimental evolution will be dominated by processes like genetic drift and possibly rare chromosomal rearrangements (vid. White 1978) to a much greater extent than is theoretically expected with moderate values of N_e. However, while there are any number of low N_e studies of experimental evolution that use sexual populations (vid. Garland and Rose 2009), for such work to achieve strong-inference status *still greater* experimental replication of populations has to be used than we have employed here. That is because such small N_e *populations* will be subject to much greater genetic drift, leading in turn to high levels of between-replicate variation, much higher than those found here for our moderate N_e populations. Therefore, in order to get appropriate statistical signal strength, much greater replication will be required for such research to achieve strong inference. This is not to argue against such research. Indeed, we have ourselves created an experimental system that features 48 such small N_e populations (Santos et al. in prep.), a system that could be profitably used to test such a small N_e scenario for the action of null mechanisms of speciation. This experimental strategy should be distinguished from the common-place use of newly-derived isofemale lines from outbreeding natural populations (e.g. Coyne and Orr 1989; 1997; Reed and Markow 2004; Koroi et al. 2006; Jennings et al. 2011; Kao et al. 2015). Such isofemale lines generally feature very low Darwinian fitness, and do not constitute particularly plausible models for speciation in most Mendelian populations. On the other hand, founder-flush experiments providing an

interesting middle ground of relevance to this context in which small N_e populations might offer more possibilities for the action of null mechanisms of speciation.

Most efforts of uniform experimental selection involved subjecting populations of *Drosophil*a or *Musca* to a series of population bottlenecks then allowing them to expand to study the effect of drift to foster prezygotic isolation (reviewed in Rice and Hostert 1993). The results of these studies are mixed with a majority of pairwise combinations showing no prezygotic isolation. The few studies that demonstrated a positive result for prezgotic isolation used populations that were produced by crossing flies collected from different locations (Dodd and Powell 1985; Ringo et al. 1985). It is not clear whether reproductive incompatibilities arose via drift or were the product of inherited variants from different locations (cf. Rundle et al. 1998). Overall these types of studies generally suffer from poor replication with just two or in some cases three replicates per treatment. In addition due to the investments of time and resources required to sustain selection the majority of published studies measure either prezygotic or postzygotic barriers, but rarely both (for review see Coyne and Orr 2004; Nosil 2012). Given the inconsistencies of past experimental approaches, founder-flush experiments might still provide a viable option to test the action of null mechanisms of speciation.

In a sense, null theories of speciation are now on probation. It is incumbent upon their proponents to show convincingly that such speciation mechanisms work *in cases where the action of ecological speciation mechanisms can be clearly precluded.* Given this constraint, we doubt that any appropriate support for most, or all, null mechanisms of speciation can be derived from studies of populations evolving in the wild. Under natural conditions, it seems to us that it would be almost prohibitively difficult to mount a plausible scientific argument that long-allopatric populations that have evolved reproductive isolation have done so in the absence of

any type of environmentally-driven difference in mechanisms of natural selection. Instead, we suggest, it is only in large-scale, well-replicated, laboratory evolution studies of small N_e that convincing corroboration of null mechanisms of speciation can be achieved.

Criticisms of our Experimental Paradigm

Though our views of what constitutes good science are largely Popperian, we are not unaware that scientists generally don't like to play by Popperian rules (vid. Kuhn 1962; Lakatos 1970; Feyerabend 1975). Thus it is important to address the maneuvers by which avid proponents of null speciation theories might seek to defend their theories against the evidence we have marshaled here.

An obvious criticism of the experiments presented here is that they depend critically on selection regimes that are evidently artificial. Indeed, we do not claim that populations maintained with specific, narrow, reproductive windows like those of our A, B, and C culture regimes are common in nature. There are cases of univoltine insect species which do have such narrow synchronized reproductive windows, like mayflies, but they are fairly unusual.

Experimental regimes which might be better suited to generalization to nature might feature adaptation to specific toxic plant hosts, as in the case of *Drosophila sechellia,* which breeds on the fallen fruit of the shrub *Morinda citrifolia,* a relatively toxic fruit before it begins to ferment (R'kha et al. 1991; Legal et al. 1992; Legal et al. 1994; Farine et al. 1996; Amlou et al. 1998). Adaptation to such a specific substrate might generally produce the kind of ecological speciation that we have found here, in a relatively natural manner.

Thus one might imagine emulating such a substrate-specific pattern of ecological differentiation in laboratory evolution project with *Drosophila.* From what we have seen of rapid responses to directional selection in this fruit fly genus (vid. Rose et al. 2004; Matos et al. 2002), we believe that such a more plausible experimental paradigm might generate similar patterns to those adduced in the data of this dissertation. This might be a useful next step toward the further resolution of the competition between ecological and null speciation theories.

However, it should be borne in mind that those who defend decaying scientific theories can be remarkably versatile in their demands for more experiments that falsify their favored hypotheses (vid. Lakatos, 1970; Feyerabend, 1975), even as they cling to meager circumstantial evidence in support of those suppositions. This is not an argument against continuing to falsify the claims of those defending falsified theories; rather it is an argument to continue to do so with better and better experiments, so that more neutral third parties can see which hypotheses merit continued consideration.

Why are Null Hypotheses for Speciation so Common?

As we ourselves have been convinced of the merits of null theories of allopatric speciation in the past, an interesting question is why so many evolutionary geneticists have proposed such theories. Certainly direct evidence in favor of such theories has not generally been abundant, although sympatric speciation by autopolyploidization and allopolyploidization is an indubitable phenomenon, particularly in plants (Stebbins 1950; Grant 1981; Ramsey and Schemske 2002) But the idea that multiple allopatric populations might evolve reproductive

isolation *without the involvement of environmentally-driven natural selection* has been both recurrent since 1900, yet lacks convincing empirical support.

Our guess is that one reason for the enduring popularity of null mechanisms of speciation is the "classical" tradition in evolutionary genetics (vid. Lewontin 1974). Before 1966, this tradition was based on the supposition that there is extremely little segregating genetic variation in natural populations. Since the discovery of abundant genetic variation in outbreeding natural populations, from humans (Harris 1966) to fruit flies (Hubby and Lewontin 1966), a neoclassical paradigm has since formed which modifies the strict classical paradigm by admitting the existence of both neutral and nearly-neutral standing genetic variation (e.g. Kimura 1983). This now-dominant paradigm supposes that selectively maintained genetic polymorphism is quite rare, with evolutionary change of functional phenotypes driven by rare selective substitutions (vid. Rose et al. 2011; Burke, 2012). In such an evolutionary context, it seems entirely plausible that comparably rare genomic rearrangements or transposable element invasions might instead be the key drivers of reproductive isolation (e.g. Rose and Doolittle, 1983).

But there is an alternative, now minority, paradigm for evolutionary genetics, in which abundant genetic variation is maintained by selection at many loci across the genome (Rose et al. 2011). With this evolutionary genetic paradigm, it is expected that the abundant genome-wide genetic variation that is commonly detected in natural populations (e.g. Mackay et al. 2012) will include many sites in the genome that impinge on functional characters (vid. Burke et al. 2010). With this paradigm, there is nothing surprising about the rapid functional phenotypic and genome-wide that is now commonly detected in studies of *Drosophila* experimental evolution (Burke et al. 2010; Turner et al. 2011; Orozco-ter Wengel et al. 2012).

In effect, the results found here with respect to the rapid action of laboratory selection in producing incipient reproductive isolation is another component of a mounting body of evidence against the applicability of the neoclassical paradigm, at least with respect to evolution in outbreeding Mendelian populations. We do not dispute that clonal evolution with its characteristic purging of genetic variation during selection will produce evolutionary scenarios fully in keeping with the neoclassical paradigm (e.g. Tenaillon et al. 2012). But those are not evolutionary settings in which allopatric speciation is relevant.

Conclusion

We have attempted to put our research findings in a more general context in this Chapter. In particular, in view of the extensive evidence against null theories of speciation, we have given extensive attention to plausible defensive maneuvers that might be attempted by defenders of such theories. We have also, to a limited extent, placed the present research in the larger context of research on the evolutionary genetics of outbreeding Mendelian species. In our opinion, many widespread views concerning the evolutionary genetics of speciation have been influenced, perhaps unduly, but the now-dominant neoclassical consensus concerning the genetic foundations of adaptation in Mendelian populations. We ourselves were among those who were so influenced. But the mounting evidence against the neoclassical paradigm of adaptation that is coming from studies of functional and genomic responses to directional selection is now joined by the less direct evidence against it provided by the present dissertation. Our research supports the causal predominance of natural selection in establishing reproductive isolation.

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APPENDIX A

Components of Reproductive Isolation for Test-crosses in Chapter 4

Table A4.1. Results of the mate-choice assay for prezygotic reproductive isolation among populations invading A-type environments.

Regime-Spoke	Scenario	Female	Male 1	Male 2	Total Matings		*p*-value
					Male 1	Male 2	
A-1	Null	ACO_1	ACO_1	ACO_2	27	32	0.515
	Null Conv.	ACO_1	ACO_1	AO_1	29	20	0.199
	Ecological	ACO_1	ACO_1	CO_1	29	23	0.405
	Interaction	ACO_1	ACO_1	CO_2	21	22	0.879
	Null	ACO_2	ACO_1	ACO_2	26	23	0.668
	Null Conv.	AO_1	ACO_1	AO_1	24	22	0.768
	Ecological	CO_1	ACO_1	CO_1	16	35	0.008*
	Interaction	CO_2	ACO_1	CO_2	13	32	0.005*
A-2	Null	ACO_2	ACO_2	ACO_3	25	28	0.680
	Null Conv.	ACO_2	ACO_2	AO_2	31	23	0.276
	Ecological	ACO_2	ACO_2	CO_2	26	24	0.777
	Interaction	ACO_2	ACO_2	CO_3	39	11	<.0001*
	Null	ACO_3	ACO_2	ACO_3	26	28	0.785
	Null Conv.	AO_2	ACO_2	AO_2	35	22	0.085
	Ecological	CO_2	ACO_2	CO_2	15	33	0.009*
	Interaction	CO_3	ACO_2	CO_3	16	33	0.015*
A-3	Null	ACO_3	ACO_3	ACO_4	25	28	0.680
	Null Conv.	ACO_3	ACO_3	AO_3	33	23	0.181
	Ecological	ACO_3	ACO_3	CO_3	29	30	0.896
	Interaction	ACO_3	ACO_3	CO_4	30	26	0.593
	Null	ACO_4	ACO_3	ACO_4	20	26	0.376
	Null Conv.	AO_3	ACO_3	AO_3	28	20	0.248
	Ecological	CO_3	ACO_3	CO_3	11	23	0.040*
	Interaction	CO_4	ACO_3	CO_4	20	37	0.024*
A-4	Null	ACO_4	ACO_4	ACO_5	27	26	0.891
	Null Conv.	ACO_4	ACO_4	AO_4	22	23	0.881
	Ecological	ACO_4	ACO_4	CO_4	24	22	0.768
	Interaction	ACO_4	ACO_4	CO_5	22	18	0.527
	Null	ACO_5	ACO_4	ACO_5	22	16	0.330
	Null Conv.	AO_4	ACO_4	AO_4	26	29	0.686
	Ecological	CO_4	ACO_4	CO_4	13	28	0.019*
	Interaction	CO_5	ACO_4	CO_5	16	33	0.015*
A-5	Null	ACO_5	ACO_5	ACO_1	22	22	1.000
	Null Conv.	ACO_5	ACO_5	AO_5	28	27	0.893
	Ecological	ACO_5	ACO_5	CO_5	28	25	0.680
	Interaction	ACO_5	ACO_5	CO_1	23	27	0.572
	Null	ACO_1	ACO_5	ACO_1	23	34	0.145
	Null Conv.	AO_5	ACO_5	AO_5	21	29	0.258
	Ecological	CO_5	ACO_5	CO_5	4	25	<.0001*
	Interaction	CO_1	ACO_5	CO_1	17	38	0.005*

Table A4.2. Results of the mate-choice assay for prezygotic reproductive isolation among populations invading C-type environments.

Regime -Spoke	Scenario	Female	Male 1	Male 2	Matings		*p*-value
					Male 1	Male 2	
C-1	Null	CO_1	CO_1	CO_2	27	25	0.782
	Null Conv.	CO_1	CO_1	nCO_1	31	25	0.423
	Ecological	CO_1	CO_1	ACO_1	43	15	0.0002*
	Interaction	CO_1	CO_1	ACO_2	41	16	0.001*
	Null	CO_2	CO_1	CO_2	25	32	0.354
	Null Conv.	nCO_1	CO_1	nCO_1	32	26	0.431
	Ecological	ACO_1	CO_1	ACO_1	24	23	0.884
	Interaction	ACO_2	CO_1	ACO_2	25	33	0.294
C-2	Null	CO_2	CO_2	CO_3	29	25	0.586
	Null Conv.	CO_2	CO_2	nCO_2	17	37	0.006*
	Ecological	CO_2	CO_2	ACO_2	41	14	0.0003*
	Interaction	CO_2	CO_2	ACO_3	33	18	0.036*
	Null	CO_3	CO_2	CO_3	21	30	0.208
	Null Conv.	nCO_2	CO_2	nCO_2	25	23	0.773
	Ecological	ACO_2	CO_2	ACO_2	25	27	0.782
	Interaction	ACO_3	CO_2	ACO_3	21	33	0.102
C-3	Null	CO_3	CO_3	CO_4	22	24	0.768
	Null Conv.	CO_3	CO_3	nCO_3	25	23	0.773
	Ecological	CO_3	CO_3	ACO_3	29	14	0.022*
	Interaction	CO_3	CO_3	ACO_4	25	12	0.033*
	Null	CO_4	CO_3	CO_4	24	20	0.546
	Null Conv.	nCO_3	CO_3	nCO_3	23	25	0.773
	Ecological	ACO_3	CO_3	ACO_3	17	18	0.866
	Interaction	ACO_4	CO_3	ACO_4	18	21	0.631
C-4	Null	CO_4	CO_4	CO_5	27	30	0.691
	Null Conv.	CO_4	CO_4	nCO_4	20	29	0.199
	Ecological	CO_4	CO_4	ACO_4	31	13	0.007*
	Interaction	CO_4	CO_4	ACO_5	29	14	0.022*
	Null	CO_5	CO_4	CO_5	25	31	0.423
	Null Conv.	nCO_4	CO_4	nCO_4	32	25	0.354
	Ecological	ACO_4	CO_4	ACO_4	22	30	0.267
	Interaction	ACO_5	CO_4	ACO_5	26	30	0.593
C-5	Null	CO_5	CO_5	CO_1	30	28	0.793
	Null Conv.	CO_5	CO_5	nCO_5	35	17	0.013*
	Ecological	CO_5	CO_5	ACO_5	38	12	0.0002*
	Interaction	CO_5	CO_5	ACO_1	37	18	0.01*
	Null	CO_1	CO_5	CO_1	35	22	0.090
	Null Conv.	nCO_5	CO_5	nCO_5	26	30	0.593
	Ecological	ACO_5	CO_5	ACO_5	32	23	0.225
	Interaction	ACO_1	CO_5	ACO_1	31	26	0.508

Table A4.3. Components of reproductive isolation for test-crosses under the A-type environments. Isolation components vary from zero (no barrier) to one (complete isolation). (F) indicates choosing female. Asterisks indicate p-values below .05.

ACO_1 Spoke: Reproductive Isolation Values						
Pop. Cross R.I. Barrier	Pop 1.	R.I.V.	*p*-value	Pop 2.	R.I.V.	*p*-value
ACO_2 ➔ ACO_1						
Ancestor k_x	ACO_2	0.113	0.765	ACO_1	-	-
Ancestor Dev.	ACO_2	0.119	0.006*	ACO_1	-	-
Mate-choice	ACO_2 (F)	-0.130	0.668	ACO_1 (F)	-0.185	0.515
Cohabiting k_x	c A_{21}	-0.031	0.999	c A_{12}	0.140	0.564
F_1 Hybrid Dev.	F_1 A_{21}	-0.049	0.679	F_1 A_{12}	-0.073	0.325
F_1 Hybrid k_x	F_1 A_{21}	0.087	0.907	F_1 A_{12}	0.161	0.400
F_2 Hybrid Dev.	F_2 A_{21}	0.112	0.012*	F_2 A_{12}	0.078	0.231
AO_1 ➔ ACO_1						
Ancestor k_x	AO_1	0.269	0.06	ACO_1	-	-
Ancestor Dev.	AO_1	-0.049	0.999	ACO_1	-	-
Mate-choice	AO_1 (F)	-0.091	0.768	ACO_1 (F)	0.310	0.199
Cohabiting k_x	c AO-A_{11}	0.389	0.001*	c A-AO_{11}	0.084	0.954
F_1 Hybrid Dev.	F_1 AO-A_{11}	0.227	0.384	F_1 A-AO_{11}	0.053	0.998
F_1 Hybrid k_x	F_1 AO-A_{11}	0.161	0.550	F_1 A-AO_{11}	-0.237	0.136
F_2 Hybrid Dev.	F_2 AO-A_{11}	0.498	0.0003*	F_2 A-AO_{11}	0.462	0.001*
CO_1 ➔ ACO_1						
Ancestor k_x	CO_1	-0.200	0.679	ACO_1	-	-
Ancestor Dev.	CO_1	1.000	<0.0001*	ACO_1	-	-
Mate-choice	CO_1 (F)	0.543	0.008*	ACO_1 (F)	0.207	0.879
Cohabiting k_x	c CO-A_{11}	0.157	0.531	c A-CO_{11}	0.149	0.098
F_1 Hybrid Dev.	F_1 CO-A_{11}	0.859	<0.0001*	F_1 A-CO_{11}	0.416	<0.0001*
F_1 Hybrid k_x	F_1 CO-A_{11}	-0.093	0.925	F_1 A-CO_{11}	-0.409	0.903
F_2 Hybrid Dev.	F_2 CO-A_{11}	0.967	<0.0001*	F_2 A-CO_{11}	0.886	<0.0001*
CO_2 ➔ ACO_1						
Ancestor k_x	CO_2	-0.040	0.996	ACO_1	-	-
Ancestor Dev.	CO_2	1.000	<0.0001*	ACO_1	-	-
Mate-choice	CO_2 (F)	0.594	0.005*	ACO_1 (F)	-0.048	0.879
Cohabiting k_x	c CO-A_{21}	-0.256	0.999	c A-CO_{12}	-0.597	0.098
F_1 Hybrid Dev.	F_1 CO-A_{21}	0.817	<0.0001*	F_1 A-CO_{12}	0.358	<0.0001*
F_1 Hybrid k_x	F_1 CO-A_{21}	0.131	0.980	F_1 A-CO_{12}	-0.192	0.903
F_2 Hybrid Dev.	F_2 CO-A_{21}	0.927	<0.0001*	F_2 A-CO_{12}	0.858	<0.0001*

Table A4.4. Components of reproductive isolation for test-crosses under the A-type environments. Isolation components vary from zero (no barrier) to one (complete isolation). (F) indicates choosing female. Asterisks indicate p-values below .05.

ACO_2 Spoke: Reproductive Isolation Values						
Pop. Cross R.I. Barrier	Pop 1.	R.I.V.	*p*-value	Pop 2.	R.I.V.	*p*-value
ACO_3 ➔ ACO_2						
Ancestor k_x	ACO_3	0.181	0.627	ACO_2	-	-
Ancestor Dev.	ACO_3	0.101	0.823	ACO_2	-	-
Mate-choice	ACO_3 (F)	0.071	0.785	ACO_2 (F)	-0.120	0.680
Cohabiting k_x	c A_{32}	-0.001	1.000	c A_{23}	-0.264	0.209
F_1 Hybrid Dev.	F_1 A_{32}	0.254	0.019*	F_1 A_{23}	0.048	0.992
F_1 Hybrid k_x	F_1 A_{32}	0.061	0.995	F_1 A_{23}	0.191	0.573
F_2 Hybrid Dev.	F_2 A_{32}	0.188	0.183	F_2 A_{23}	0.117	0.713
AO_2 ➔ ACO_2						
Ancestor k_x	AO_2	-0.262	0.887	ACO_2	-	-
Ancestor Dev.	AO_2	0.039	0.668	ACO_2	-	-
Mate-choice	AO_2 (F)	-0.591	0.085	ACO_2 (F)	0.258	0.276
Cohabiting k_x	c AO-A_{22}	-0.783	0.022*	c A-AO_{22}	-0.516	0.307
F_1 Hybrid Dev.	F_1 AO-A_{22}	-0.013	0.988	F_1 A-AO_{22}	0.031	0.796
F_1 Hybrid k_x	F_1 AO-A_{22}	0.148	0.992	F_1 A-AO_{22}	0.129	0.996
F_2 Hybrid Dev.	F_2 AO-A_{22}	0.015	0.988	F_2 A-AO_{22}	0.086	0.008*
CO_2 ➔ ACO_2						
Ancestor k_x	CO_2	-0.146	0.608	ACO_2	-	-
Ancestor Dev.	CO_2	1.000	<0.0001*	ACO_2	-	-
Mate-choice	CO_2 (F)	0.545	0.009*	ACO_2 (F)	0.077	0.777
Cohabiting k_x	c CO-A_{22}	0.066	0.945	c A-CO_{22}	0.161	0.498
F_1 Hybrid Dev.	F_1 CO-A_{22}	0.633	<0.0001*	F_1 A-CO_{22}	0.434	<0.0001*
F_1 Hybrid k_x	F_1 CO-A_{22}	0.258	0.058	F_1 A-CO_{22}	0.348	0.002*
F_2 Hybrid Dev.	F_2 CO-A_{22}	0.9	<0.0001*	F_2 A-CO_{22}	0.721	<0.0001*
CO_3 ➔ ACO_2						
Ancestor k_x	CO_3	-0.140	0.769	ACO_2	-	-
Ancestor Dev.	CO_3	1.000	<0.0001*	ACO_2	-	-
Mate-choice	CO_3 (F)	0.515	0.015*	ACO_2 (F)	0.718	<.0001*
Cohabiting k_x	c CO-A_{32}	-0.086	0.899	c A-CO_{23}	0.086	0.900
F_1 Hybrid Dev.	F_1 CO-A_{32}	0.676	<0.0001*	F_1 A-CO_{23}	0.611	<0.0001*
F_1 Hybrid k_x	F_1 CO-A_{32}	0.291	0.005*	F_1 A-CO_{23}	0.162	0.348
F_2 Hybrid Dev.	F_2 CO-A_{32}	0.906	<0.0001*	F_2 A-CO_{23}	0.804	<0.0001*

Table A4.5. Components of reproductive isolation for test-crosses under the A-type environments. Isolation components vary from zero (no barrier) to one (complete isolation). (F) indicates choosing female. Asterisks indicate p-values below .05.

ACO_3 Spoke: Reproductive Isolation Values						
Pop. Cross R.I. Barrier	Pop 1.	R.I.V.	*p*-value	Pop 2.	R.I.V.	*p*-value
ACO_4 ➔ ACO_3						
Ancestor k_x	ACO_4	-0.421	0.019*	ACO_3	-	-
Ancestor Dev.	ACO_4	-0.015	1.000	ACO_3	-	-
Mate-choice	ACO_4 (F)	0.231	0.376	ACO_3 (F)	-0.120	0.680
Cohabiting k_x	c A_{43}	0.181	0.747	c A_{34}	-0.249	0.413
F_1 Hybrid Dev.	F_1 A_{43}	-0.132	0.603	F_1 A_{34}	-0.171	0.231
F_1 Hybrid k_x	F_1 A_{43}	-0.130	0.924	F_1 A_{34}	0.062	0.997
F_2 Hybrid Dev.	F_2 A_{43}	-0.174	0.231	F_2 A_{34}	0.058	0.968
AO_3 ➔ ACO_3						
Ancestor k_x	AO_3	-0.194	0.934	ACO_3	-	-
Ancestor Dev.	AO_3	-0.231	0.015*	ACO_3	-	-
Mate-choice	AO_3 (F)	-0.400	0.248	ACO_3 (F)	0.303	0.181
Cohabiting k_x	c AO-A_{33}	-0.009	1.000	c A-AO_{33}	-0.314	0.642
F_1 Hybrid Dev.	F_1 AO-A_{33}	-0.254	0.004*	F_1 A-AO_{33}	-0.263	0.003*
F_1 Hybrid k_x	F_1 AO-A_{33}	0.034	1.000	F_1 A-AO_{33}	-0.034	1.000
F_2 Hybrid Dev.	F_2 AO-A_{33}	-0.212	0.038*	F_2 A-AO_{33}	-0.045	0.992
CO_3 ➔ ACO_3						
Ancestor k_x	CO_3	-0.051	1.000	ACO_3	-	-
Ancestor Dev.	CO_3	1.000	<0.0001*	ACO_3	-	-
Mate-choice	CO_3 (F)	0.522	0.040*	ACO_3 (F)	-0.034	0.896
Cohabiting k_x	c CO-A_{33}	0.153	0.947	c A-CO_{33}	0.138	0.967
F_1 Hybrid Dev.	F_1 CO-A_{33}	0.960	<0.0001*	F_1 A-CO_{33}	0.724	<0.0001*
F_1 Hybrid k_x	F_1 CO-A_{33}	0.363	0.285	F_1 A-CO_{33}	0.393	0.202
F_2 Hybrid Dev.	F_2 CO-A_{33}	0.892	<0.0001*	F_2 A-CO_{33}	0.786	<0.0001*
CO_4 ➔ ACO_3						
Ancestor k_x	CO_4	0.013	1.000	ACO_3	-	-
Ancestor Dev.	CO_4	1.000	<0.0001*	ACO_3	-	-
Mate-choice	CO_4 (F)	0.459	0.024*	ACO_3 (F)	0.133	0.593
Cohabiting k_x	c CO-A_{43}	0.069	0.995	c A-CO_{34}	0.232	0.466
F_1 Hybrid Dev.	F_1 CO-A_{43}	0.800	<0.0001*	F_1 A-CO_{34}	0.723	<0.0001*
F_1 Hybrid k_x	F_1 CO-A_{43}	0.654	<0.0001*	F_1 A-CO_{34}	0.291	0.213
F_2 Hybrid Dev.	F_2 CO-A_{43}	0.914	<0.0001*	F_2 A-CO_{34}	0.875	<0.0001*

Table A4.6. Components of reproductive isolation for test-crosses under the A-type environments. Isolation components vary from zero (no barrier) to one (complete isolation). (F) indicates choosing female. Asterisks indicate p-values below .05.

ACO_4 Spoke: Reproductive Isolation Values						
Pop. Cross R.I. Barrier	Pop 1.	R.I.V.	*p*-value	Pop 2.	R.I.V.	*p*-value
ACO_5 ➔ ACO_4						
Ancestor k_x	ACO_5	-0.151	0.981	ACO_4	-	-
Ancestor Dev.	ACO_5	0.074	0.919	ACO_4	-	-
Mate-choice	ACO_5 (F)	-0.375	0.330	ACO_4 (F)	0.037	0.891
Cohabiting k_x	c A_{54}	-0.062	1.000	c A_{45}	0.145	0.984
F_1 Hybrid Dev.	F_1 A_{54}	-0.020	1.000	F_1 A_{45}	0.099	0.752
F_1 Hybrid k_x	F_1 A_{54}	0.146	0.984	F_1 A_{45}	-0.317	0.675
F_2 Hybrid Dev.	F_2 A_{54}	0.304	0.001*	F_2 A_{45}	0.051	0.979
AO_4 ➔ ACO_4						
Ancestor k_x	AO_4	-0.160	0.433	ACO_4	-	-
Ancestor Dev.	AO_4	-0.188	0.034*	ACO_4	-	-
Mate-choice	AO_4 (F)	0.103	0.686	ACO_4 (F)	-0.045	0.881
Cohabiting k_x	c AO-A_{44}	0.474	<0.0001*	c A-AO_{44}	-0.226	0.093
F_1 Hybrid Dev.	F_1 AO-A_{44}	0.171	0.062	F_1 A-AO_{44}	-0.117	0.458
F_1 Hybrid k_x	F_1 AO-A_{44}	-0.329	0.002*	F_1 A-AO_{44}	0.207	0.160
F_2 Hybrid Dev.	F_2 AO-A_{44}	0.338	<0.0001*	F_2 A-AO_{44}	0.232	0.003*
CO_4 ➔ ACO_4						
Ancestor k_x	CO_4	-0.148	1.000	ACO_4	-	-
Ancestor Dev.	CO_4	1.000	<0.0001*	ACO_4	-	-
Mate-choice	CO_4 (F)	0.536	0.019*	ACO_4 (F)	0.083	0.768
Cohabiting k_x	c CO-A_{44}	0.144	0.996	c A-CO_{44}	0.066	1.000
F_1 Hybrid Dev.	F_1 CO-A_{44}	0.981	<0.0001*	F_1 A-CO_{44}	0.8	<0.0001*
F_1 Hybrid k_x	F_1 CO-A_{44}	0.244	0.954	F_1 A-CO_{44}	-0.246	0.953
F_2 Hybrid Dev.	F_2 CO-A_{44}	0.966	<0.0001*	F_2 A-CO_{44}	0.898	<0.0001*
CO_5 ➔ ACO_4						
Ancestor k_x	CO_4	-0.104	0.934	ACO_4	-	-
Ancestor Dev.	CO_4	1.000	<0.0001*	ACO_4	-	-
Mate-choice	CO_4 (F)	0.515	0.015*	ACO_4 (F)	0.182	0.527
Cohabiting k_x	c CO-A_{54}	0.091	0.956	c A-CO_{45}	0.048	0.998
F_1 Hybrid Dev.	F_1 CO-A_{54}	0.998	<0.0001*	F_1 A-CO_{45}	0.923	<0.0001*
F_1 Hybrid k_x	F_1 CO-A_{54}	0.006	1.000	F_1 A-CO_{45}	0.07	0.988
F_2 Hybrid Dev.	F_2 CO-A_{54}	0.875	<0.0001*	F_2 A-CO_{45}	0.924	<0.0001*

Table A4.7. Components of reproductive isolation for test-crosses under the A-type environments. Isolation components vary from zero (no barrier) to one (complete isolation). (F) indicates choosing female. Asterisks indicate p-values below .05.

ACO_5 Spoke: Reproductive Isolation Values						
Pop. Cross R.I. Barrier	Pop 1.	R.I.V.	*p*-value	Pop 2.	R.I.V.	*p*-value
ACO_1 ➔ ACO_5						
Ancestor k_x	ACO_1	0.132	0.935	ACO_5	-	-
Ancestor Dev.	ACO_1	-0.020	0.998	ACO_5	-	-
Mate-choice	ACO_1 (F)	0.324	0.145	ACO_5 (F)	0.000	1.000
Cohabiting k_x	c A_{15}	-0.310	0.227	c A_{51}	0.086	0.990
F_1 Hybrid Dev.	F_1 A_{15}	-0.102	0.46	F_1 A_{51}	0.168	0.018*
F_1 Hybrid k_x	F_1 A_{15}	0.033	1.000	F_1 A_{51}	-0.214	0.639
F_2 Hybrid Dev.	F_2 A_{15}	0.076	0.671	F_2 A_{51}	-0.032	0.988
AO_5 ➔ ACO_5						
Ancestor k_x	AO_5	-0.106	0.947	ACO_5	-	-
Ancestor Dev.	AO_5	0.267	0.0003*	ACO_5	-	-
Mate-choice	AO_5 (F)	0.276	0.258	ACO_5 (F)	0.036	0.893
Cohabiting k_x	c AO-A_{55}	-0.156	0.771	c A-AO_{55}	0.08	0.985
F_1 Hybrid Dev.	F_1 AO-A_{55}	-0.045	0.980	F_1 A-AO_{55}	-0.084	0.808
F_1 Hybrid k_x	F_1 AO-A_{55}	-0.603	<0.0001*	F_1 A-AO_{55}	-0.662	<0.0001*
F_2 Hybrid Dev.	F_2 AO-A_{55}	0.115	0.467	F_2 A-AO_{55}	-0.003	1.000
CO_5 ➔ ACO_5						
Ancestor k_x	CO_5	-0.178	0.414	ACO_5	-	-
Ancestor Dev.	CO_5	1.000	<0.0001*	ACO_5	-	-
Mate-choice	CO_5 (F)	0.840	<.0001*	ACO_5 (F)	0.107	0.680
Cohabiting k_x	c CO-A_{55}	0.111	0.853	c A-CO_{55}	0.094	0.920
F_1 Hybrid Dev.	F_1 CO-A_{55}	0.928	<0.0001*	F_1 A-CO_{55}	0.807	<0.0001*
F_1 Hybrid k_x	F_1 CO-A_{55}	0.303	0.0178*	F_1 A-CO_{55}	0.456	<0.0001*
F_2 Hybrid Dev.	F_2 CO-A_{55}	0.958	<0.0001*	F_2 A-CO_{55}	0.945	<0.0001*
CO_1 ➔ ACO_5						
Ancestor k_x	CO_1	-0.133	0.894	ACO_5	-	-
Ancestor Dev.	CO_1	1.000	<0.0001*	ACO_5	-	-
Mate-choice	CO_1 (F)	0.553	0.005*	ACO_5 (F)	-0.174	0.572
Cohabiting k_x	c CO-A_{15}	-0.094	0.952	c A-CO_{51}	0.115	0.892
F_1 Hybrid Dev.	F_1 CO-A_{15}	0.856	<0.0001*	F_1 A-CO_{51}	0.778	<0.0001*
F_1 Hybrid k_x	F_1 CO-A_{15}	0.224	0.292	F_1 A-CO_{51}	0.43	0.0001*
F_2 Hybrid Dev.	F_2 CO-A_{15}	0.968	<0.0001*	F_2 A-CO_{51}	0.941	<0.0001*

Table A4.8. Components of reproductive isolation for test-crosses under the C-type environments. Isolation components vary from zero (no barrier) to one (complete isolation). (F) indicates choosing female. Asterisks indicate p-values below .05.

CO_1 Spoke: Reproductive Isolation Values						
Pop. Cross R.I. Barrier	Pop 1.	R.I.V.	*p*-value	Pop 2.	R.I.V.	*p*-value
CO_2 ➔ CO_1						
Ancestor k_x	CO_2	0.125	0.980	CO_1	-	-
Mate-choice	CO_2 (F)	0.219	0.354	CO_1 (F)	0.074	0.782
Cohabiting k_x	c C_{21}	-0.228	0.787	c C_{12}	-0.271	0.637
F_1 Hybrid k_x	F_1 C_{21}	-0.236	0.761	F_1 C_{12}	-0.325	0.435
nCO_1 ➔ CO_1						
Ancestor k_x	nCO_1	0.082	0.951	CO_1	-	-
Mate-choice	nCO_1 (F)	-0.231	0.431	CO_1 (F)	0.194	0.423
Cohabiting k_x	c nCO-C_{11}	-0.162	0.501	c C-nCO_{11}	-0.165	0.48
F_1 Hybrid k_x	F_1 nCO-C_{11}	-0.234	0.118	F_1 C-nCO_{11}	-0.13	0.725
ACO_1 ➔ CO_1						
Ancestor k_x	ACO_1	0.786	<0.0001*	CO_1	-	-
Mate-choice	ACO_1 (F)	-0.043	0.884	CO_1 (F)	0.651	0.0002*
Cohabiting k_x	c ACO-C_{11}	0.745	<0.0001*	c C-ACO_{11}	-0.165	0.713
F_1 Hybrid k_x	F_1 ACO-C_{11}	-0.069	0.991	F_1 C-ACO_{11}	-0.222	0.393
ACO_2 ➔ CO_1						
Ancestor k_x	ACO_2	0.715	<0.0001*	CO_1	-	-
Mate-choice	ACO_2 (F)	0.242	0.294	CO_1 (F)	0.61	0.001*
Cohabiting k_x	c ACO-C_{21}	0.837	<0.0001*	c C-ACO_{12}	0.01	1.000
F_1 Hybrid k_x	F_1 ACO-C_{21}	0.012	1.000	F_1 C-ACO_{12}	-0.066	0.990

Table A4.9. Components of reproductive isolation for test-crosses under the C-type environments. Isolation components vary from zero (no barrier) to one (complete isolation). (F) indicates choosing female. Asterisks indicate p-values below .05.

CO_2 Spoke: Reproductive Isolation Values						
Pop. Cross R.I. Barrier	Pop 1.	R.I.V.	*p*-value	Pop 2.	R.I.V.	*p*-value
CO_3 ➔ CO_2						
Ancestor k_x	CO_3	0.023	1.000	CO_2	-	-
Mate-choice	CO_3 (F)	0.300	0.208	CO_2 (F)	0.138	0.586
Cohabiting k_x	c C_{32}	0.065	0.999	c C_{23}	-0.117	0.990
F_1 Hybrid k_x	F_1 C_{32}	0.061	1.000	F_1 C_{23}	-0.272	0.710
nCO_2 ➔ CO_2						
Ancestor k_x	nCO_2	-0.304	0.938	CO_2	-	-
Mate-choice	nCO_2 (F)	-0.087	0.773	CO_2 (F)	-1.176	0.006*
Cohabiting k_x	c nCO-C_{22}	-0.385	0.844	c C-nCO_{22}	0.316	0.926
F_1 Hybrid k_x	F_1 nCO-C_{22}	-0.176	0.995	F_1 C-nCO_{22}	-0.202	0.990
ACO_2 ➔ CO_2						
Ancestor k_x	ACO_2	0.758	0.046*	CO_2	-	-
Mate-choice	ACO_2 (F)	0.074	0.782	CO_2 (F)	0.659	0.0003*
Cohabiting k_x	c ACO-C_{22}	0.908	0.008*	c C-ACO_{22}	-0.231	0.952
F_1 Hybrid k_x	F_1 ACO-C_{22}	0.285	0.889	F_1 C-ACO_{22}	0.169	0.988
ACO_3 ➔ CO_2						
Ancestor k_x	ACO_3	0.857	<0.0001*	CO_2	-	-
Mate-choice	ACO_3 (F)	0.364	0.102	CO_2 (F)	0.455	0.036*
Cohabiting k_x	c ACO-C_{32}	0.974	<0.0001*	c C-ACO_{23}	0.216	0.837
F_1 Hybrid k_x	F_1 ACO-C_{32}	0.589	0.014*	F_1 C-ACO_{23}	0.257	0.709

Table A4.10. Components of reproductive isolation for test-crosses under the C-type environments. Isolation components vary from zero (no barrier) to one (complete isolation). (F) indicates choosing female. Asterisks indicate p-values below .05.

CO_3 Spoke: Reproductive Isolation Values						
Pop. Cross R.I. Barrier	Pop 1.	R.I.V.	*p*-value	Pop 2.	R.I.V.	*p*-value
CO_4 ➔ CO_3						
Ancestor k_x	CO_4	0.222	0.854	CO_3	-	-
Mate-choice	CO_4 (F)	-0.200	0.546	CO_3 (F)	-0.091	0.768
Cohabiting k_x	c C_{43}	0.017	1.000	c C_{34}	-0.079	0.998
F_1 Hybrid k_x	F_1 C_{43}	0.291	0.648	F_1 C_{34}	0.261	0.745
nCO_3 ➔ CO_3						
Ancestor k_x	nCO_3	0.134	0.994	CO_3	-	-
Mate-choice	nCO_3 (F)	0.080	0.773	CO_3 (F)	0.080	0.773
Cohabiting k_x	c nCO-C_{33}	0.061	1.000	c C-nCO_{33}	0.000	1.000
F_1 Hybrid k_x	F_1 nCO-C_{33}	0.155	0.988	F_1 C-nCO_{33}	0.191	0.970
ACO_3 ➔ CO_3						
Ancestor k_x	ACO_3	0.602	<0.0001*	CO_3	-	-
Mate-choice	ACO_3 (F)	0.056	0.866	CO_3 (F)	0.517	0.022*
Cohabiting k_x	c ACO-C_{33}	0.811	<0.0001*	c C-ACO_{33}	-0.071	0.986
F_1 Hybrid k_x	F_1 ACO-C_{33}	0.294	0.074	F_1 C-ACO_{33}	-0.012	1.000
ACO_4 ➔ CO_3						
Ancestor k_x	ACO_4	0.748	<0.0001*	CO_3.	-	-
Mate-choice	ACO_4 (F)	0.143	0.631	CO_3 (F)	0.520	0.033*
Cohabiting k_x	c ACO-C_{43}	0.856	<0.0001*	c C-ACO_{34}	-0.135	0.772
F_1 Hybrid k_x	F_1 ACO-C_{43}	0.052	0.996	F_1 C-ACO_{34}	-0.036	0.999

Table A4.11. Components of reproductive isolation for test-crosses under the C-type environments. Isolation components vary from zero (no barrier) to one (complete isolation). (F) indicates choosing female. Asterisks indicate p-values below .05.

CO_4 Spoke: Reproductive Isolation Values						
Pop. Cross R.I. Barrier	Pop 1.	R.I.V.	*p*-value	Pop 2.	R.I.V.	*p*-value
CO_5 ➔ CO_4						
Ancestor k_x	CO_5	0.257	0.905	CO_4	-	-
Mate-choice	CO_5 (F)	0.194	0.423	CO_4 (F)	-0.111	0.691
Cohabiting k_x	c C_{54}	-0.076	1.000	c C_{45}	0.217	0.952
F_1 Hybrid k_x	F_1 C_{54}	0.129	0.995	F_1 C_{45}	0.036	1.000
nCO_4 ➔ CO_4						
Ancestor k_x	nCO_4	0.274	0.707	CO_4	-	-
Mate-choice	nCO_4 (F)	-0.28	0.354	CO_4 (F)	-0.450	0.199
Cohabiting k_x	c nCO-C_{44}	-0.069	0.999	c C-nCO_{44}	-0.001	1.000
F_1 Hybrid k_x	F_1 nCO-C_{44}	0.418	0.244	F_1 C-nCO_{44}	0.235	0.823
ACO_4 ➔ CO_4						
Ancestor k_x	ACO_4	0.824	<0.0001*	CO_4	-	-
Mate-choice	ACO_4 (F)	0.267	0.267	CO_4 (F)	0.581	0.007*
Cohabiting k_x	c ACO-C_{44}	0.794	<0.0001*	c C-ACO_{44}	-0.256	0.563
F_1 Hybrid k_x	F_1 ACO-C_{44}	-0.088	0.993	F_1 C-ACO_{44}	-0.159	0.909
ACO_5 ➔ CO_4						
Ancestor k_x	ACO_5	0.833	<0.0001*	CO_4	-	-
Mate-choice	ACO_5 (F)	0.133	0.593	CO_4 (F)	0.517	0.022*
Cohabiting k_x	c ACO-C_{54}	0.841	<0.0001*	c C-ACO_{45}	-0.213	0.625
F_1 Hybrid k_x	F_1 ACO-C_{54}	0.026	1.000	F_1 C-ACO_{45}	0.116	0.958

Table A4.12. Components of reproductive isolation for test-crosses under the C-type environments. Isolation components vary from zero (no barrier) to one (complete isolation). (F) indicates choosing female. Asterisks indicate p-values below .05.

CO_5 Spoke: Reproductive Isolation Values						
Pop. Cross R.I. Barrier	Pop 1.	R.I.V.	*p*-value	Pop 2.	R.I.V.	*p*-value
CO_1 ➔ CO_5						
Ancestor k_x	CO_1	-0.305	0.021*	CO_5	-	-
Mate-choice	CO_1 (F)	-0.591	0.090	CO_5 (F)	0.067	0.793
Cohabiting k_x	c C_{15}	0.253	0.096	c C_{51}	-0.206	0.276
F_1 Hybrid k_x	F_1 C_{15}	-0.484	<0.0001*	F_1 C_{51}	-0.277	0.049*
nCO_5 ➔ CO_5						
Ancestor k_x	nCO_5	-0.284	0.291	CO_5	-	-
Mate-choice	nCO_5 (F)	0.133	0.593	CO_5 (F)	0.514	0.013*
Cohabiting k_x	c nCO-C_{55}	-0.364	0.078	c C-nCO_{55}	-0.273	0.335
F_1 Hybrid k_x	F_1 nCO-C_{55}	-0.032	1.000	F_1 C-nCO_{55}	-0.013	1.000
ACO_5 ➔ CO_5						
Ancestor k_x	ACO_5	0.653	0.005*	CO_5	-	-
Mate-choice	ACO_5 (F)	-0.391	0.225	CO_5 (F)	0.514	0.0002*
Cohabiting k_x	c ACO-C_{55}	0.873	<0.0001*	c C-ACO_{55}	-0.143	0.971
F_1 Hybrid k_x	F_1 ACO-C_{55}	0.041	1.000	F_1 C-ACO_{55}	-0.363	0.357
ACO_1 ➔ CO_5						
Ancestor k_x	ACO_1	0.744	0.010*	CO_5	-	-
Mate-choice	ACO_1 (F)	-0.192	0.508	CO_5 (F)	0.684	0.010*
Cohabiting k_x	c ACO-C_{15}	0.763	0.008*	c C-ACO_{51}	-0.126	0.993
F_1 Hybrid k_x	F_1 ACO-C_{15}	-0.354	0.601	F_1 C-ACO_{51}	-0.296	0.766