UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS
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Evolutionary Divergence Between Two Closely Related Species, *Drosophila madeirensis* and *D. subobscura*

Quantitative genetic differentiation, reproductive barriers and evolutionary potential

CARLA JOSÉ AZEVEDO REGO

DOUTORAMENTO EM BIOLOGIA
(BIOLOGIA EVOLUTIVA)

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In memory of my mother, a true “mother courage”

Em memória da minha mãe, uma verdadeira “mãe coragem”

Nota Prévia

Na elaboração da presente dissertação, e nos termos do nº 1 do Artigo 40, Capítulo V, do Regulamento de Estudos Pós-Graduados da Universidade de Lisboa, publicado no *Diário da República – II Série Nº 153*, de 5 de Julho de 2003, esclarece-se que foram usados integralmente artigos científicos já publicados (2), aceites para publicação (1) e submetidos (1), em revistas indexadas de circulação internacional, os quais integram os capítulos da presente tese. Tendo os referidos trabalhos sido realizados em colaboração, a candidata esclarece que participou integralmente no planeamento, na análise e discussão dos resultados e na elaboração de todos os trabalhos apresentados.

Contents

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"That which does not kill us makes us stronger."

Friedrich Nietzsche

RESUMO

A especiação é uma das problemáticas mais interessantes e controversas em Biologia Evolutiva e, apesar da profusão de estudos nesta área, a origem de novas espécies continua a ser 'o mistério dos mistérios' da Evolução. Estudos detalhados da diferenciação genética entre espécies próximas podem contribuir para a compreensão dos mecanismos envolvidos numa origem recente, assim como para caracterizar o seu estado actual e fazer previsões acerca da sua evolução futura. A tese de doutoramento aqui apresentada envolveu um conjunto de estudos com o objectivo de caracterizar o grau de diferenciação genética entre duas espécies estreitamente aparentadas, *Drosophila madeirensis* e *Drosophila subobscura*, nas suas diversas implicações, quer no que respeita à compreensão do seu grau de isolamento reprodutor actual quer das suas potencialidades evolutivas futuras. Nesse âmbito, é analisada a sua diferenciação genética em termos de várias características associadas à fitness, focando a atenção em possíveis indicadores de constrangimentos genéticos que dificultem o sucesso de híbridos; é analisado o seu grau de isolamento reprodutor, bem como os problemas de desenvolvimento em híbridos, em particular no que respeita ao papel de uma eventual instabilidade de desenvolvimento como barreira pós-zigótica. Finalmente, são analisadas as implicações do diferente fundo genético destas espécies na sua capacidade de se adaptarem a novos ambientes.

O **capítulo 1** consiste numa introdução geral sobre vários aspectos relacionados com o fenómeno de especiação, nomeadamente, conceitos de espécie e modelos de especiação. Em seguida são abordadas em maior detalhe as problemáticas mais directamente relacionadas com o tema da tese, especificamente a contribuição de efeitos genéticos aditivos e não-aditivos para a diferenciação entre espécies. O isolamento reprodutor é também referido nas suas vertentes pré- e pós-zigótica, sendo abordados aspectos comportamentais que impedem o acasalamento entre indivíduos de espécies diferentes e problemas apresentados pelos híbridos, tais como inviabilidade, esterilidade e anomalias morfológicas. Outra questão focada é a influência da diferenciação entre espécies no processo de adaptação ao cativeiro e as potenciais implicações em termos de conservação. Em seguida, é apresentada uma actualização sobre a biologia do modelo em estudo, com particular ênfase nos aspectos relacionados com o seu isolamento reprodutor.

No **capítulo 2** é apresentado um estudo sobre a diferenciação genética quantitativa entre *Drosophila madeirensis* e *D. subobscura*, em diversas características da história da vida, envolvendo a análise de diferenças entre as espécies parentais e os seus híbridos interespecíficos de primeira e segunda geração. Esta comparação permitiu determinar a contribuição de efeitos genéticos aditivos, de dominância, epistasia e maternos para a diferenciação entre estas duas espécies. Permitiu ainda determinar se constrangimentos genéticos derivados das diferenças

entre as espécies se manifestavam, especificamente na expressão de efeitos de dominância ou epistasia negativos.

No **capítulo 3** é apresentada uma análise comparativa de acasalamentos conspecíficos e heteroespecíficos das duas espécies, para determinar se ocorre acasalamento preferencial entre indivíduos da mesma espécie, e qual a sua possível contribuição para o isolamento reprodutor entre estas espécies.

No **capítulo 4** é abordada a questão do impacto da hibridação na estabilidade de desenvolvimento. Com esse objectivo, foram comparados os níveis de assimetria flutuante (uma medida da estabilidade do desenvolvimento) manifestados pelas espécies parentais e pelos híbridos de ambas as direcções de cruzamento. O desenho experimental utilizado neste estudo incluiu uma análise de irmãos e meios-irmãos, que possibilitou dissecar a contribuição de efeitos genéticos para os níveis de assimetria encontrados nos híbridos, permitindo assim estimar mais correctamente os níveis de estabilidade de desenvolvimento.

No **capítulo 5** é apresentado um estudo comparativo das trajectórias evolutivas de *D. madeirensis* e *D. subobscura* durante a adaptação a um novo ambiente comum. Para tal, foram fundadas populações laboratoriais de ambas as espécies, a partir de populações naturais, e foi analisada a sua taxa evolutiva durante a adaptação ao laboratório, em análises cobrindo várias gerações e diversas características da história da vida.

Finalmente, o **capítulo 6** apresenta uma discussão geral, integrando os resultados obtidos ao longo dos estudos referidos, terminando com sugestões para estudos futuros, de forma a aprofundar o conhecimento nas diversas áreas abordadas, assim como em novas linhas de investigação relacionadas com as problemáticas gerais da especiação e diferenciação genética entre espécies.

Um dos aspectos mais discutidos na diferenciação entre espécies, são os mecanismos genéticos nela envolvidos, nomeadamente a contribuição de efeitos aditivos e não aditivos. *D. madeirensis* e *D. subobscura* são duas espécies estreitamente aparentadas capazes de produzir, em laboratório, híbridos viáveis e férteis em ambas as direcções de cruzamento, sendo por isso um bom modelo de estudo para analisar neste contexto. Os resultados desta análise (capítulo 2) indicam que efeitos de dominância e epistasia negativos são importantes na diferenciação de várias características entre estas espécies. Este resultado é muito interessante, pois revela que constrangimentos derivados da divergência genética entre estas espécies poderão funcionar como barreira à introgressão genética, por baixa fitness de eventuais híbridos.

Neste trabalho caracterizamos também os comportamentos de acasalamento inter e intraespecíficos, como eventuais barreiras reprodutivas pré-zigóticas, analisando ainda a possível ocorrência de assimetrias de acasalamento nas duas direcções interespecíficas. Também averiguamos se os acasalamentos interespecíficos envolvem menor fecundidade que os intraespecíficos. Os resultados (capítulo 3) indicam que os acasalamentos intraespecíficos

ocorrem mais facilmente que os interespecíficos, tal como seria de esperar. Indicam ainda que os acasalamentos entre espécies, envolvendo fêmeas *D. subobscura* e machos *D. madeirensis*, ocorrem mais facilmente do que os acasalamentos na direcção recíproca. No entanto, esta direcção de cruzamento apresenta menor fecundidade e menor viabilidade de híbridos, sobretudo em fêmeas. Os resultados sugerem assim uma "assimetria" de barreiras reprodutoras entre cruzamentos recíprocos: essencialmente pré-zigótica na direcção fêmea *D. madeirensis* x macho *D. subobscura*, e pós-zigótica na direcção oposta. Este resultado é interessante pois contraria uma das regras mais universais em Biologia, a regra de Haldane.

O desenvolvimento dos organismos encontra-se normalmente protegido contra perturbações ambientais e genéticas através de mecanismos reguladores tais como a canalização e a estabilidade de desenvolvimento. Geralmente assume-se que a hibridação, pode quebrar complexos coadaptados de genes que evoluíram independentemente em cada espécie, perturbando os mecanismos que promovem a estabilidade do desenvolvimento. Os híbridos interespecíficos são por isso um material valioso para analisar neste contexto. Neste trabalho (capítulo 4) foi avaliado o impacto da hibridação na estabilidade de desenvolvimento, comparando os níveis de assimetria flutuante em híbridos *vs.* espécies parentais. Os resultados revelaram que apesar de as fêmeas híbridas com mãe *D. madeirensis* apresentarem uma maior assimetria no tamanho das asas, esta assimetria não se deveu a um maior ruído de desenvolvimento nos híbridos. Curiosamente, mais uma vez, foram as fêmeas híbridas que apresentaram as maiores repercussões do fenómeno de hibridação, pois os machos apresentaram níveis de assimetria semelhantes aos das espécies parentais. Assim, apesar de em laboratório a direcção de cruzamento que envolve fêmeas *D. madeirensis* e machos *D. subobscura* ser a mais prolífica em termos de híbridos, estes dados indicam a existência de uma potencial barreira pós-zigótica (que reforça a pré-zigótica antes referida), uma vez que as fêmeas híbridas apresentam uma elevada assimetria alar o que pode comprometer o seu sucesso reprodutor.

Outro aspecto interessante da diferenciação entre espécies que foi alvo deste trabalho, foi avaliar de que modo essa diferenciação influencia a adaptação a um novo ambiente. Para tal, foram mantidas em laboratório populações de ambas as espécies, em condições semelhantes, e o seu desempenho foi comparado em várias gerações ao longo do processo de adaptação ao laboratório. A expectativa mais conservativa seria a de convergência, dado o grau de proximidade das duas espécies e o facto de estarem sujeitas a pressões selectivas semelhantes. No entanto, a diferenciação ocorrida em termos genéticos durante a sua especiação poderia afectar a sua dinâmica evolutiva específica, eventualmente causando um aumento da sua divergência inicial. Ambas as espécies mostraram sinais de adaptação ao laboratório (capítulo 5). No entanto, a taxa evolutiva foi muito mais acentuada em *D. subobscura*. Tal pode estar relacionado com o facto de *D. subobscura* ser uma espécie generalista com uma vasta distribuição, presumivelmente com uma maior capacidade de adaptação, enquanto que *D.*

madeirensis é uma espécie endémica especializada nos recursos fornecidos pela *Laurissilva*. Estes resultados são relevantes em termos de Biologia Evolutiva e de Conservação. Em termos evolutivos podemos constatar que a adaptação local pode não só "reforçar" o isolamento reprodutor como constranger a possível evolução das espécies. Em termos de conservação, os nossos resultados evidenciam dificuldades de generalização em programas de conservação *ex situ*, uma vez que ilustram a importância dos fundos genéticos para os efeitos da adaptação ao cativeiro.

Os resultados apresentados nesta tese são sumarizados e integrados no capítulo final (capítulo 6), salientando-se a sua relevância não só em termos de Biologia Evolutiva, mas também de Conservação. Em termos gerais, este estudo revela a importância de uma aproximação complementar para a compreensão dos padrões e processos evolutivos envolvidos na divergência genética entre espécies. Nomeadamente, o conjunto destes estudos revelou a importância de cobrir aspectos tão diversos como a caracterização do estado actual de diferenciação genética entre as espécies e as suas implicações em termos de constrangimentos evolutivos – reflexo do seu passado como unidades evolutivas independentes. Revelou igualmente a importância da análise do seu grau de isolamento reprodutor, quer em termos comportamentais quer de desenvolvimento de híbridos – como indicador da sua manutenção como unidades evolutivas distintas. Os estudos apresentados revelam ainda a importância da análise da possível divergência genética futura entre as espécies quando em adaptação a alterações ambientais – numa projecção para o futuro destas espécies. Todos estes aspectos são também claramente relevantes em termos de conservação, dado que alterações ambientais podem alterar barreiras de isolamento reprodutor, e afectar diferencialmente as espécies, sobretudo quando estas diferem no seu grau de adaptabilidade. Consequentemente, seria extremamente interessante aplicar estas várias aproximações a outros pares de espécies próximas, para determinar em que medida se poderão generalizar os padrões observados. Estes estudos irão contribuir para aprofundar o conhecimento nesta área, ainda tão necessitada de estudos empíricos, contribuindo para uma melhor compreensão do processo de especiação, o ainda 'mistério dos mistérios' da Biologia Evolutiva.

Palavras-chave: *Drosophila*, especiação, barreiras reprodutoras, estabilidade de desenvolvimento, potencial adaptativo.

ABSTRACT

Speciation and species differentiation are very important issues in Evolutionary Biology. This thesis focuses several aspects related with the differentiation between two closely related species, *Drosophila madeirensis* and *Drosophila subosbcura*, namely the contribution of additive and non-additive genetic effects to that differentiation, the contribution of assortative mating to their reproductive isolation, the analysis of hybrid developmental problems expressed as higher fluctuating asymmetry and their underlying causes (developmental noise), and the implications of species differentiation in terms of adaptation to a novel, common environment. The results indicate that negative dominance and epistasis are both involved in the genetic differentiation between these species. Both species present assortative mating, conspecific matings being more likely. Furthermore, the two reciprocal cross directions apparently present different reproductive barriers. In the cross involving *D. madeirensis* females the barrier is mostly prezygotic, with mating being hard to observe, however, this cross direction yields a high number of hybrids with an even sex-ratio. On the other hand, mating in the reciprocal cross is easy to observe but produces fewer hybrids with a male-biased sex ratio. The analysis comparing fluctuating asymmetry levels between hybrids and parental species indicates that, although hybridization disrupts developmental buffering, hybrid females presenting higher asymmetry, this disruption does not reflect higher developmental noise, as fluctuating asymmetry levels are similar to parental species. The results comparing species differences in life history traits and evolutionary dynamics indicate that these closely related species differ in the adaptation to new conditions (captivity). These findings have important implications for several fields, namely Evolutionary Biology, Speciation, Development and Conservation, which are discussed at the end of this thesis.

Keywords: *Drosophila*, speciation, reproductive barriers, developmental stability, adaptive potential.

Chapter 1.

GENERAL INTRODUCTION

"I look at the term species, as one arbitrarily given for the sake of convenience to a set of individuals closely resembling each other...." Charles Darwin, 1859

"The species problem is like a sword, thrust by Darwin into the stone, and left for us to yank upon with determination…" J. Hey, 2001

Species and their formation always played an important role in Biology. Species are one of the fundamental units of comparison in almost all fields of Biology, from Anatomy to Behaviour, including Ecology, Taxonomy and Conservation Biology, among others. In part, the importance of species derives from Systematics which established the taxonomic framework used in all areas of Biology, even before Darwin and his celebrated work '*On the Origin of Species*'.

With the emergence of Evolutionary Biology species became of even more central importance, being the level of organization where the two major forces of evolution intercross and interact: microevolutionary changes within populations (anagenesis) and genetic differentiation among populations, leading ultimately to the diversity among species that we see today (cladogenesis). Whatever the definition used, species are definitely of central importance in Evolutionary Biology (e.g. Coyne and Orr, 2004; de Queiroz, 2005).

Despite the importance of species, much debate still goes on around species concepts, about the evolutionary mechanisms that originate them – speciation – and about what maintains them as evolutionary units. Finally, much is still to be learned about species differences, not only about what exactly those differences are and how they arose, but also in what way they affect the future evolutionary processes of species, leading or not to further divergence. These are some of the issues we will address

below, after which we will define how we intend to contribute to the debate with the present thesis.

1.1. Species concepts

What is a species? This is one of the more everlasting questions in Biology. Traditionally, in every known culture, man instinctively classifies natural diversity into categories, grouping the organisms found in nature according with their degree of similarity. However, biologists have to find a general effective definition which can be tested (Freeman and Herron, 2004). Linnaeus and other early taxonomists grouped individuals according with their morphological similarity using a "typological" or "essentialist" notion of species (Mayr, 1963). However, this presented some problems, such as ascribing males and females from the same species to different species due to their morphological differences. Another problem with this definition is the potential to underestimate species numbers due to the existence of "sister species" morphologically undistinguishable.

The publication of "*On the Origin of Species*" by Charles Darwin in 1859, changed the way we think about species. He introduced the notion that evolution, by means of natural selection and adaptation, shapes natural diversity and that species are not static entities conforming to a type, but present natural variation and are constantly adapting and evolving. Since then, many definitions of "species" have been proposed. However, we still lack a definite, unique definition (e.g. Futuyma, 1998; Hey, 2001; Coyne and Orr, 2004; Freeman and Herron, 2004; de Queiroz, 2005; Mallet, 2006a).

Generally speaking species are the smallest evolutionary independent units, consisting of interbreeding populations that evolve independently from other populations. The problem with species definitions is establishing general practical criteria for identifying populations (or groups of populations) that are evolving independently (Freeman and Herron, 2004).

One of the most influential species definitions was proposed by Ernst Mayr in 1942 and is known as The Biological Species Concept. According with this definition reproductive isolation is the criterion to establish evolutionary independency. Consequently, species are "groups of populations that interbreed or have the potential to do so, which are reproductively isolated from other such groups". If populations do not

hybridize or do not produce fertile progeny when they interbreed they belong to different species. However, this view of species does not apply to asexual populations and can not be tested in the fossil record (Futuyma, 1998; Freeman and Herron, 2004). Another instance where this definition does not apply, is when two different species can produce viable and fertile hybrids as it happens with some bird and plant species (Grant and Grant, 1992; Arnold, 1997; Price and Bouvier, 2002). However, according with Coyne and Orr (2004), there is not sufficient evidence to support that natural hybridization can pose a problem for the Biological Species Concept.

The Phylogenetic Species Concept (Cracaft, 1989), uses monophyly as a criterion to identify species, monophyletic groups being taxa that contain all the known descendents of a single common ancestor. Under this concept, species are identified estimating the phylogeny of closely related populations and finding the smallest monophyletic groups (Futuyma, 1998; Freeman and Herron, 2004; de Queiroz, 2005; Mallet, 2006a). However, few phylogenies are available for use in this sense, due to the high costs involved both in terms of money and time to properly estimate evolutionary relationships (Freeman and Herron, 2004). Consequently this criterion is impractical for many species groups.

Many other species definitions have been proposed, some examples being the Ecological Species Concept (Van Valen, 1976), the Evolutionary Species Concept (Simpson, 1951) and the Cohesion Species Concept (Templeton, 1998). However, despite the large amount of species concepts, defining species according with the degree of similarity between individuals is still useful. The Morphospecies Concept is mostly employed by taxonomists, being used in fossils and in groups where estimating reproductive isolation is inviable or when well estimated phylogenies are not available (as it happens in most cases). It has also been used in conservation and biodiversity studies analysing highly diverse groups such as insects. Its use facilitates and accelerates the time consuming process of sorting and identifying species, aggravated by the scarcity of specialists for some taxonomic groups (e.g. Olivier and Beattie, 1996; Primack, 1998). The advantage of this criterion is the ease to apply it, but its improper use can easily lead to a confounding effect, in extreme cases making it impossible to compare species classifications made by different investigators. Moreover, this criterion fails to identify cryptic species which are morphologically very similar but differ in

ecology, courtship behaviour or other characteristics which effectively isolate them reproductively (Freeman and Herron, 2004).

As we have seen, defining what a species is, it is not a simple task. Many species definitions have been proposed but neither gives the definitive answer. Perhaps there will never be a "definitive" definition as Darwin (1859) seems to have foreseen by admitting that species may be as unreal and artificial categories as genera. In fact, according with Mallet (2001, 2006a), in recent years the reality of species has been challenged by several authors. However, Coyne and Orr (2004) argue that the reality of species is reinforced by the high concordance between the species classifications done by layman from several different cultures and scientists. And even Darwin (1859) recognized that although there is no consensual definition of species, everyone knows what they are talking about when they speak of species.

Overall the term "species" has two meanings and uses in Biology, which can overlap, but are distinct. One more related with biology and evolution, embodied by the Biological Species Concept, the other serving merely classification purposes. For many sexually reproducing organisms both meanings overlap, but for non-sexually reproducing organisms, like bacteria, the species name only corresponds to a classification. However, despite all the controversy, defining species is useful to understand biological diversity and its origins. And, whatever the chosen definition, all evolutionary biologists agree that species play a central role in the hierarchy of life, one related with a particular evolutionary status. It is thus of utmost importance to understand what are the evolutionary mechanisms that make different populations deserve to be considered, at some point in their evolution, as distinct species.

1.2. Speciation models

A central question in Evolutionary Biology is: How are species formed? Darwin (1859) was one of the first to tackle this question, but almost 150 years later we still lack a general model of speciation that explains this process in full. Darwin contended that natural selection was the main mechanism involved in population differentiation and hence in speciation. Since Darwin many models of speciation have been proposed

and the attention shifted from natural selection to other mechanisms such as genetic drift acting in isolated populations.

Speciation, the origin of two species from a common ancestral species, can be seen as the evolution of reproductive barriers preventing gene flow between new emerging taxa (e.g. Futuyma, 1998; Turelli *et al.*, 2001). The Biological Species Concept is implicit in this definition of speciation; in fact, this concept of species is adopted by many speciation models.

Traditionally, speciation models have been classified according with the degree of geographic isolation involved: allopatric speciation with complete isolation, peripatric speciation or founder-event speciation involving peripheral populations of a species range, parapatric speciation with some degree of overlap in population distribution during divergence, and finally sympatric speciation where populations diverge in the complete absence of geographic isolation.

One of the most influential models of speciation was proposed by Mayr (1942, 1963), the Allopatric Speciation Model. In this model reproductive isolation evolves in allopatry, with physical barriers preventing gene flow between populations. The lack of gene flow allows allopatric populations to diverge by any evolutionary force, with pre- and post-zygotic isolating mechanisms arising as inevitable by-products of genetic divergence (Turelli *et al*., 2001). Traditionally the speciation process has been divided in three stages: a first step, where populations become isolated (e.g. due to a geographic barrier); a second step of divergence in some traits, namely habitat use or mating behaviour; and finally a third step leading to reproductive isolation when differentiated populations contact each other. According to this model the first two stages occur while the populations are geographically isolated, while the final step occurs when secondary contact between the populations takes place. However this view of speciation, has been challenged by recent studies, namely there is increasing evidence that the first two stages of this model can take place while populations are not geographically isolated (e.g. Mallet, 2001; Turelli *et al.*, 2001; Via, 2001). There is also evidence that in many cases the last phase (secondary contact) does not occur (e.g. Freeman and Herron, 2004).

One of the most important hypotheses proposed by Mayr (1963) is that speciation is most likely to occur in small populations, that become physically isolated in the peripheral range of a species (e.g. Freeman and Herron, 2004; Orr, 2005).

Geographical isolation can arise due to dispersion and colonization of new habitats, a typical example being the amazing Hawaiian *Drosophila* radiation, with 500 described species and many more waiting to be described. In this case there is strong evidence that dispersal to new habitats triggered speciation, with small populations or even single fertilized females colonizing new islands or habitats and originating new species (DeSalle and Giddings, 1986).

Another way in which populations can become geographically isolated is through vicariance, when their natural range is divided by a new barrier, like a new emerging mountain range. One example of this involves the speciation of shrimp species following the closure of the Panama Isthmus about 3 million years ago, which separated the shrimp populations into Pacific and Atlantic populations giving rise to different species in both sides of the Isthmus (Knowlton *et al.*, 1993).

After isolation has taken place, either by dispersion or vicariance, the next stage is divergence. Divergence between populations can be caused by genetic drift, its effects being more marked in small populations. Traditionally, drift has been seen as an essential part of the second stage in speciation. However, this view has been controversial (Freeman and Herron, 2004). For instance, Lande (1980, 1981) has shown that when populations suffer bottlenecks only rare alleles are lost by drift. According with this author, significant changes in allele frequency due to drift, are only possible in extremely small populations that remain small for a considerable amount of time. Another problem with viewing drift as a main cause of divergence and speciation has been stressed by Grant and Grant (1996), who pointed out that in the last 150 years human activities have led to the introduction of numerous small populations in new habitats, but neither of these introductions resulted in speciation events. Nowadays, evolutionary biologists tend to consider natural selection as the most important force promoting population divergence (e.g. Mallet, 2001; Turelli *et al.*, 2001; Via, 2001; Coyne and Orr, 2004; Freeman and Herron, 2004; Orr, 2005), leading to a more Darwinian view of speciation.

In parapatric speciation new species evolve from contiguous populations rather than separate populations. According with Turelli and co-workers (2001), given a sufficient broad geographical range, any mechanism that can originate species in allopatry can also cause divergence in parapatry. In a species with a wide distribution, if different alleles arise in different locations of the species range, and if these prove

incompatible with each other when they meet, they will contribute to reproductive isolation in parapatry. Reproductive isolation might arise as a by product of local adaptation, just as in allopatric speciation. The "isolation by distance" is necessary for parapatric speciation and depends on the strength of selection acting on population divergence. If strong selection occurs, either causing local adaptation or maintaining alternative peaks, then divergence leading to reproductive isolation is possible in a small spatial scale. The existence of narrow clines and hybrid zones reveals that selection can overcome gene flow over a small spatial scale, allowing parapatric speciation (Turelli *et al.*, 2001).

Sympatric speciation is another example of divergence without geographic isolation, involving the emergence of two species from a single population, without the aid of allopatry. This is one of the most controversial models of speciation. For 60 years it was practically ignored and deemed implausible, the argument being that continuous gene flow would prevent the establishment of fixed genetic differences, necessary for species formation (e.g. Mayr, 1963; Futuyma, 1998; Coyne and Price, 2000; Johannesson, 2001; Via, 2001; Gavrilets, 2003). However, recently this type of speciation has been the target of renewed interest and efforts have been made to test it empirically (e.g. Meyer *et al.*, 1990; Via, 2001; Barluenga and Meyer, 2004; Bush and Butlin, 2004; Savolainen *et al.*, 2006).

In particular, Bush and Butlin (2004), defend that sympatric divergence due to host-shift could be the major source of diversity in many phytophagous and parasitoid insects which are particularly species-rich. Colonizing a new host and specializing in its resources could lead to speciation, particularly if the insects mate assortatively within their host. *Rhagoletis pomonella* flies seem to be an example of such ongoing divergence. Flies from this species specialized in two different fruits (apples and hawthorn), mate on or near the fruits, and differ genetically according with the type of fruit they use (e.g. Feder *et al.*, 1990). Apples are clearly a new resource for these flies, as apple trees were introduced from Europe less than three centuries ago, and both hawthorn trees and *Rhagoletis* are native to North America. However, recent evidences have cast some doubt whether this particular divergence can be seen entirely as an example of sympatric speciation (Jiggins and Bridle, 2004). It seems that at least some of the genetic variability that allowed the host-shift to apples, derived from pre-existing geographical variation (Feder *et al*., 2003; Xie *et al.*, 2007).

Cichlids from African and Nicaraguan crater lakes are other potential examples where sexual selection and ecology possibly drove sympatric speciation (e.g. Meyer *et al.*, 1990; Alphen *et al*., 2004; Barluenga and Meyer, 2004; Barluenga *et al*., 2006). Another recent example of sympatric divergence concerns two endemic species of palm trees from Lord Howe Island in Western Australia (e.g. Savolainen *et al.*, 2006). In this case, divergence could be due to an association with different soil types, with repercussions in flowering time and consequently in reproductive isolation (Savolainen *et al*., 2006).

Demonstrating sympatric speciation is difficult, as claims of this mode of speciation must demonstrate species sympatry, sister relationships, reproductive isolation, and exclude an earlier allopatric phase (Coyne and Orr, 2004). This last possibility has proven particularly hard to rule out completely. For example, it could be argued that the two palm tree species mentioned above resulted from two independent colonization events from the same source population, one colonization originating one species and the other the second species. Posterior hybridization between them could lead to both species being more similar to each other than to the ancestral species/population giving the wrong impression that they are sister species (Blackman, 2006).

For some authors, what is perceived as sympatric speciation is in fact microallopatric speciation (Coyne and Price, 2000). The argument is that, in spite of no physical barrier being involved, such as a mountain range, geographic isolation can in fact be involved, if individuals have low mobility and are associated with different habitats/hosts, feeding and mating on or near them.

However, despite all the controversies, there is one undisputed scenario of sympatric isolation: mutations causing polyploidy (e.g. Mayr, 1947; Ramsey and Schemske, 1998). Polyploidy can result in instant reproductive isolation between parental and daughter populations, due to gametic incompatibilities caused by differences in chromosome number. In spite of the few studies relating phylogeny and polyploidy there is some indication that this mechanism can play an important role in speciation, especially in plants (Freeman and Herron, 2004), but also in some animals (e.g. Alves *et al.,* 2001; Keller and Gerhardt, 2001).

As we have seen, several models or modes of speciation have been proposed. Some are consensual like allopatric speciation or by polyploidy but others are still

controversial, like sympatric speciation. However, there is no simple explanation for all the "endless forms" we see in nature. Some authors defend that in some cases the divergence can be caused by a mixture of several speciation modes originating speciation mode plurality (Xie *et al.*, 2007). In spite of all the knowledge gathered on species formation, for the time being speciation remains "the mystery of mysteries" as Darwin had already called it.

1.3. Genetic differentiation between species

Honest differences are often a healthy sign of progress. Mahatma Gandhi

Population differentiation is one of the most important aspects in evolutionary biology, since it is responsible for evolutionary diversity and ultimately for the evolution of different species. The genetic processes involved in divergence are particularly interesting and have been amply debated. Much of the disagreement surrounding this issue concerns the role that epistasis may play in population evolution (e.g. Barton and Turelli, 1989; Whitlock *et al*., 1995; Fenster *et al.*, 1997).

The Dobzhansky-Muller model states that, given enough time, population divergence leads to the independent accumulation of co-adapted gene complexes in different lineages. The interbreeding of different lineages would lead to the disruption of these complexes, resulting in hybrid incompatibilities and consequently leading to lower hybrid fitness (e.g. Turelli and Orr, 2000; Coyne and Orr, 2004). These incompatibilities may range from developmental problems expressed as morphological abnormalities to more extreme effects like hybrid sterility or inviability (e.g. Arnold, 1997; Dowling and Secor, 1997; Coyne and Orr, 2004).

Although the Dobzhansky-Muller model is generally accepted to explain present differentiation and hybrid incompatibilities, the role that gene interaction may play in general evolutionary terms, and in speciation in particular, is highly controversial (Coyne *et al*., 1997, 2000; Wade and Goodnight, 1998; Goodnight and Wade, 2000; Gravilets, 2004). Namely, it is not clear whether it is a cause of population divergence, like Wright defended in his Shifting Balance theory (Wright, 1977), or just a consequence of the divergence process. Wright's adaptive landscape with fitness peaks

and valleys relies on gene interaction. In this scenario limited gene flow between populations or demes originates population structure making the evolution of coadapted gene combinations more likely. Small 'population' sizes would also contribute to the genetic differentiation, fostering the process. However, if epistasis is not a relevant component in the genetic basis of differentiation of fitness related traits, then the Shifting Balance theory is not needed to explain population evolution and a Fisherian scenarium of selection is enough to lead populations to higher fitness peaks (Fenster *et al.*, 1997).

This standing controversy leads to the need to determine and quantify the role of epistatic effects in population evolution. Several methods have been used to detect and measure the effects of epistasis on evolution (reviewed in Fenster *et al.*, 1997). Among these, hybrid breakdown is probably the most powerful method to establish the contribution of epistasis to present differentiation (Coyne *et al.*, 1997; Fenster *et al.*, 1997). This approach involves the comparison of the means of different crosses (generations) between different populations or species (crosses within populations or species and their comparison with several generations of hybrid crosses, e.g. F1 and F2 hybrids, and eventually with backcrosses, among hybrids and their parental populations/species). Consequently, this procedure detects the eventual reduction of fitness caused by the disruption of coadapted gene complexes, being a direct test of the contribution of epistasis to differentiation (Whitlock *et al.*, 1995).

Crossing different lineages can have unpredictable consequences. In some cases it may produce hybrids fitter than both parental types, known as hybrid vigour or heterosis; while in others it may lead to the opposite outcome - hybrid breakdown (Lynch and Walsh, 1998). Hybrid vigour has been known by animal and plant breeders for a very long time and has had important repercussions in agriculture and livestock breeding (Darwin, 1876; Dodds, 1955; Donald, 1955; Arnold, 1997). Hybrid breakdown is frequent in nature (e.g. Breeuwer and Werren, 1995; Arnold, 1997; Lynch and Walsh, 1998; Burke and Arnold, 2001), and its relative frequency has led to the traditional view that hybrids are "evolutionary dead ends" (Mayr, 1963).

Hybrid vigour and hybrid breakdown are both determined by non-additive genetic effects, dominance (within locus) and epistasis (among loci) respectively. While evidences of dominance are abundant, epistasis has proven harder to find (see Lynch and Walsh, 1998). Moreover, the epistatic effects found are not very consistent. For

instance, most studies involve several intraspecific crosses, and only a fraction of these crosses indicates epistasis (e.g. Lair *et al.*, 1997; Gilchrist and Partridge, 1999; Bieri and Kawecki, 2003; Teotónio *et al.*, 2004). Also, in studies that involved several traits, only some revealed epistasis (e.g. Macnair and Cumbs, 1989; Edmands, 1999; Carrol *et al.*, 2001, 2003; Teotónio *et al*., 2004).

The difficulty in detecting epistasis is in part due to the need to use demanding designs, namely it is necessary to use hybrids of more than one generation (F1, F2 and backcrosses) (Lynch & Walsh, 1998). One problem is that, though a higher divergence time between populations/species increases the chance of gene interaction being involved in the differentiation, it also increases the probability of reproductive isolation. Reproductive isolation can, by definition, prevent the formation of the hybrid generations (F1, F2 hybrids and backcrosses) necessary to determine the relative contribution of additive and non-additive effects to the differentiation observed between species/populations (see Mather and Jinks, 1982). However, despite all the inherent difficulties, a few interspecific studies have also found evidences of epistasis, both in plants (Macnair and Cumbs, 1989; Fritz *et al.*, 2003), and animals (Breeuwer and Werren, 1995; Hatfield, 1997).

Provided the constraints mentioned above are not impeditive, Mather and Jinks (1982) present a method to dissect and quantify the several genetic effects using line mean comparisons. This method attributes different coefficients according with the effects that can be detected in each generation (type of cross), allowing the dissection of additive, dominance and epistatic effects. Furthermore, epistatic effects can be dissected into several types of digenic interactions (additive x additive, additive x dominance and dominance x dominance), when F2 hybrids and backcrosses are available.

In the absence of all the necessary generations to test for all genetic parameters, it is still possible to estimate the significance of several genetic effects using the linear relationships involving generation means (see Mather and Jinks, 1982). When testing for the genetic effects involved in species differentiation, the simplest model is tested first, and if this proves insufficient to explain the observed variation, then additional parameters are added one by one and the adequacy of the subsequent models is tested.

The simplest model to explain species differentiation involves only additive effects. Comparing both parental species gives an estimation of the differentiation

between them, and consequently is an estimate of additive effects (Kearsey and Pooni, 1996). A purely additive model predicts that F1 hybrids will be at the midpoint between both parentals. Thus deviations from this expectation indicate that dominance effects can be involved. However this does not exclude the possible involvement of other non-additive effects (Kearsey and Pooni, 1996).

The next step is to test for conformity to an additive-dominance model. Given the assumption that additive and dominance are the only effects present, the expectation is that the F2 will deviate from the mid-parental value by half the difference that separates F1 from the mid-parental value (Mather and Jinks, 1982). Any deviation from this expectation indicates that the simple model of additive plus dominance is not sufficient to explain the observed variation and allows the inference that other effects, such as epistasis, are involved.

Maternal genetic effects are also easy to test through a simple comparison of generation means. The differences in mean phenotypes of daughters from the two reciprocal F1 crosses, provide an estimate of the difference between maternal effects associated with each parental species (Lynch and Walsh, 1998). That is, if F1 hybrids differ significantly from F1 hybrids from the reciprocal cross, maternal genetic effects are involved.

The study of the genetic differentiation between species is a very interesting field in evolutionary biology. Interspecifc studies involving species with incomplete reproductive isolation provide a valuable tool to dissect the several potential genetic effects involved in that differentiation and to better understand the speciation processes involved in species formation (**see below and chapter 2**).

1.4. Reproductive isolation

A great marriage is not when the 'perfect couple' comes together. It is when an imperfect couple learns to enjoy their differences. Dave Meurer, "*Daze of Our Wives"*

Implicit in the notion of the Biological Species Concept and in most speciation models is the importance of reproductive isolation for population divergence and

ultimately for the formation of new species (speciation). However, in spite of the importance of reproductive isolation and hybrid unfitness to population differentiation, it is unlikely that genes involved in reproductive isolation are directly selected in order to prevent gene flow (Mallet, 2006b). The general view is that reproductive isolation is not directly selected, but is rather a side-effect of genetic differentiation driven by selection on other traits (e.g. Rice and Salt, 1990; Schluter, 2001).

Speciation can occur when divergent selection in contrasting environments, leads directly or indirectly to the evolution of reproductive isolation between populations. Studies involving differentiated selection lines provided empirical evidences that this type of speciation is possible (e.g. Rice and Salt, 1990; reviewed in Rice and Hostert, 1993), reinforcing the idea that this type of speciation can happen in allopatry (different selection regimes). But it is also possible in sympatry, if the selection is strong enough or if gene flow between diverging populations is low (Turelli *et al.*, 2001; Via, 2001).

Rice and Hostert (1993) reviewed laboratory studies on reproductive barriers and concluded that speciation is quite likely in situations where there is strong divergent selection relative to gene flow. They found that situations where selection promotes divergence in an adaptive trait that has additional effects on reproductive isolation (one trait-models) were particularly supportive of sympatric speciation. Examples of ecological divergence and speciation have been growing in recent years (e.g. Bush and Butlin, 2004; Rundle and Schluter, 2004; Waser and Campbell, 2004; Nosil and Crespi, 2006). It might occur indirectly as a consequence of natural selection acting on morphological, physiological or behavioural traits, or it might include reinforcement, which is direct selection on premating isolation.

Reproductive isolation can take many forms. Reproductive barriers can be broadly defined as any mechanism preventing gene flow between populations. Usually, they are divided in two main categories: prezygotic barriers, which prevent fertilization, and consequently hybrid formation, and postzygotic barriers, which reduce hybrid fitness, preventing gene flow after fertilization has taken place (e.g. Futuyma, 1998; Gavrilets, 2004).

1.4.1. Prezygotic barriers – assortative mating

Prezygotic barriers include several mechanisms preventing mating, like: temporal isolation, where different species are reproductively active at different times (e.g. different seasons); habitat or resource isolation, where differences in habitat choice or resource use prevent meetings between potential reproductive partners; ethological isolation, where differences in courtship behaviour prevent mating and mechanical isolation, resulting from morphological incompatibilities between male and female genitalia preventing mating, etc. (Futuyma, 1998; Coyne and Orr, 2004).

Prezygotic isolation also includes barriers acting after mating has taken place but before fertilization occurs. In heterospecific matings, postcopulatory-prezygotic incompatibilities between the male ejaculate and female reproductive tract can lead to reduced fertilization (Markow *et al.*, 2007). Recent evidences on reproductive morphology and biochemistry diversity in *Drosophila* indicate that they may be important in the speciation of this genus.

Several mechanisms can prevent or reduce fertilization after mating has taken place (reviewed in Markow *et al.*, 2007). For example: mating duration could be insufficient for effective sperm transfer, fertilization could express conspecific sperm precedence (e.g. Gregory and Howard, 1994) with eggs being fertilized preferentially by conspecific sperm, foreign sperm can fail to stimulate egg laying (Herndon and Wolfner, 1995), differences in sperm length and sperm positioning within the egg can also act as reproductive barriers in some *Drosophila* species (Snook, 1997).

When heterospecific individuals meet and there is a possibility they will mate, one of the first reproductive barriers to "act" is preferential mating between individuals. Positive assortative mating is an important type of prezygotic isolation. This isolating barrier involves preferential mating between conspecifics or individuals from the same population. It can either result from ecological requirements, like phytophagous insects that mate on their host plant, or from differences in courtship behaviour. Mating discrimination may be an important mechanism of reproductive isolation, and may evolve in order to prevent the costs of mating with an unsuitable partner, particularly when populations can frequently meet. Heterospecific matings can have several fitness costs, particularly for the sex with the greatest reproductive investment.

One potential cost of heterospecific matings is increased female mortality. For example, the lack of fit between male and female genitalia can cause body perforation and consequently female death. It can also damage male genitalia, impairing future matings and thus reducing male fitness (Sota and Kubota, 1998). Reduced oviposition can be another consequence in this type of matings, females mated with heterospecific males laying fewer eggs than females mated conspecifically (e.g. Wade *et al.*, 1994; Shapiro, 2000; Price *et al.*, 2001).

One of the more detrimental costs of mating with an individual from a different species is the production of maladapted hybrids. Frequently hybrids between species are less fit than parental species, ranging from being “intermediates” in ecological requirements and thus not fit in either parental habitat, to more severe consequences such as sterility or inviability, which has led to the traditional view that hybrids are evolutionary “dead ends” (Mayr, 1963). Incidentally, the bad reputation that hybrids have starts in the very word: the Latin “hybrida” derives from the Greek “hubris”, meaning "arrogance or insolence against the gods" (Schilthuizen, 2002).

Traditionally the relevance of hybridization to population evolution is recognized by botanists but almost ignored by zoologists. Hybridization is an important speciation mechanism in plants (e.g. Arnold, 1997; Rieseberg, 1997). Differentiation due to hybridization is rarer in animals but its occurrence has also been recorded (e.g. Alves *et al.*, 2001; Gompert *et al.*, 2006). Furthermore, there are also evidences that in many cases reproductive isolation is incomplete, and that hybridization and introgression are frequent both in animals and plants (e.g. Grant and Grant, 1996; Arnold, 1997; Mallet, 2005). This supports the view that hybridization can play a relevant role as an evolutionary mechanism (Barton, 2001; Coyne and Orr, 2004).

As we have seen, mating with heterospecifics can have several fitness costs, but it can also have some benefits (Wiley *et al.*, 2007). For example, heterospecific males could be more proficient in parental care than conspecific ones, reducing the costs for females. Other benefits could result from hybridization if the two species extract different resources from their environments, allowing heterospecific parents to provide a wider diversity of food resources to their offspring. According with Wiley and coworkers (2007) this would be beneficial for both parents and it could be important in reducing selection against hybridization. Females could also benefit from heterospecific

mating if conspecific males have a lower ability to obtain better territories (Wiley *et al.*, 2007).

Speciation studies on prezygotic barriers have focussed mainly on behaviour or ecological aspects preventing mating in general terms. However, several *Drosophila* species present interspecific mating asymmetry, i.e. one cross direction being easier to obtain (e.g. Kaneshiro, 1976), which represents a possibility for gene flow between species. Despite the indication that heterospecific matings can result in reduced fecundity in several organisms (as mentioned above), this type of potential barrier has been little explored in *Drosophila*. *Drosophila madeirensis* and *D. subobscura*, two closely related species with the ability to interbreed is an ideal system to test this type of potential barrier **(see below and Chapter 3)**.

1.4.2. Postzygotic barriers

Postzygotic barriers act after fertilization has taken place and reduce hybrid fitness. They can be further characterized as either extrinsic or intrinsic (Turelli *et al.*, 2001). In extrinsic isolation the relative viability and fertility of hybrids is determined by the environment in which they are tested, whereas intrinsic isolation is determined by developmental problems relatively independent from the environment. One example of environment-dependent hybrid fitness has been found in sticklebacks, where hybrids show reduced fitness when tested in either parental environment but have normal fitness when tested in the lab (Hatfield and Schluter, 1999). Ecological dependent hybrid fitness has also been found in Darwin finches (Grant and Grant, 1992) and tephritid flies (Craig *et al.*, 1997). In the following section we will pay more attention to intrinsic postzygotic barriers, the focus of some of our own studies.

1.4.2.1. Intrinsic isolation – Haldane's rule

Intrinsic isolation usually takes the form of hybrid sterility or inviability and can be found in many species pairs. Haldane's rule one of the most pervasive tenets in biology is associated with intrinsic isolation. It states that 'When in the F_1 offspring of two different animal races one sex is absent, rare, or sterile, that sex is the heterozygous

[heterogametic] sex' (Haldane, 1922). Many explanations have been proposed to elucidate Haldane's rule. According with Coyne and Orr (2004) only four remain viable to give a general explanation of this rule: the dominance theory, the faster male theory, the faster X theory and the meiotic drive theory.

The dominance theory states that Haldane's rule is caused by X-linked alleles that act recessively in hybrids. In the faster-male theory the argument is that hybrid genetic incompatibilities develop faster in males than in females. Consequently, during introgression, chromosome regions transposed from one species to another should contain more alleles related with male sterility than with female sterility. The faster X theory contends that X-linked genes have a large effect on hybrid fitness, subsequently heterogametic hybrids suffer more the negative effects of hybridization than homogametic ones. Finally, the meiotic drive theory states that Haldane's rule is caused by selfish genetic elements, which distort sex-ratios. Each species/population has its own elements and there is strong selection to suppress this drive so that normal sex-ratios are expressed. When individuals from two populations interbreed, the masked meiotic drive will be expressed in the hybrids, leading to male sterility. According with Coyne and Orr (2004) the evidence supports that dominance and faster male evolution are the main mechanisms causing Haldane's rule.

Haldane's rule is mostly found in recently diverged species, as crosses between more diverged species tend to produce inviable or sterile individuals from both sexes (e.g. Coyne and Orr, 1989, 1997; Futuyma, 1998). According with Coyne and Orr (1989) in *Drosophila* prezygotic isolation evolves faster than postzygotic isolation in sympatric species, but not in allopatric ones. This study also points out that hybrid males are affected first, in accordance with Haldane's rule, the earliest trait to arise being sterility followed by inviability. However, this can not be generalized to other groups of organisms. For example in sympatric bird species sterility evolves faster, first in males then in females, followed by inviability affecting first females and then males (Price and Bouvier, 2002).

Several studies have analysed the genetic basis of hybrid problems, uncovering several potential genes involved in the incompatibilities determining male sterility (reviewed in Coyne and Orr, 2004). Contrasting with the amount of information available on the genetic basis of hybrid sterility little is known about the developmental basis of hybrid problems. For example *Drosophila* interspecific hybrids can present

several morphological abnormalities, some examples being: differences in male genitalia (Coyne, 1983; Laurie *et al.*, 1997), in ovariole number (R' Kha, 1991), in body pigmentation (Llopart *et al.*, 2002), and extra sex combs (e.g. Papaceit *et al.*, 1991; Khadem and Krimbas, 1997).

1.4.2.2. Hybrid morphological abnormalities – developmental instability

Morphological abnormalities can be an indicator that hybridization has detrimental effects on the developmental buffering mechanisms that protect organisms against developmental perturbations. Canalization and developmental stability are two subcategories of such buffering mechanisms. Canalization reflects the genome's ability to reduce phenotypic variation due to genetic or environmental disturbances (Waddington, 1942). Canalization can constrain the evolution of buffered traits but it can also hide additional variability, repressing the expression of new mutations. Later if the canalization system breaks down, for example due to a change in selective pressures or to the mixture of different gene pools, this "hidden" variability may be expressed and this can even lead to evolutionary divergence (Gibson and Wagner, 2000). In relation to the causes of phenotypic variation a distinction between genetic and environmental canalization is necessary (Sterns *et al.*, 1995; Wagner *et al.*, 1997). Environmental canalization will tend to reduce the sensitivity of an optimized trait to environmental perturbations (Wagner *et al.*, 1997). On the other hand genetic canalization will buffer developmental pathways against the tendency of new alleles to make nonoptimal phenotypes (Gibson and Wagner, 2000).

Developmental stability, another type of buffering system, comprises a series of mechanisms that reduce the effects of developmental noise and ensure that a trait develops according with its genetic basis. Developmental noise reflects random errors of development, deriving from the stochastic nature of cellular processes. These errors disturb the patterns of cell division, differentiation and growth involved in the development of morphological structures (Klingenberg, 2004). Canalization is generally evaluated estimating interindividual variance, while fluctuating asymmetry is the most common estimator of developmental stability in bilaterally symmetric organisms. Fluctuating asymmetry is the intraindividual variation due to random differences between left and right sides in bilateral organisms.

As mentioned above, developmental noise derives from the stochastic nature of cellular processes during development. Most of these processes act locally, so a disturbance will only affect a small part on one body side (except during early embryonic development), and perturbation effects will accumulate separately in left and right sides of organs and morphological structures (see Klingenberg, 2003). In the absence of compensatory mechanisms, both sides will develop differently, and the resulting morphological asymmetry will be the visible expression of developmental noise.

Little is known about the genetic basis underlying the buffering mechanisms canalization and developmental stability. Particularly, it remains an open question whether they are partially overlapping, and share at least in part a common genetic basis (e.g. Dworkin, 2005; Santos *et al*., 2005). Several authors consider that developmental stability is a particular case of canalization (e.g. Clarke, 1998; Klingenberg and McIntyre, 1998; Meiklejohn and Hartl, 2002). For instance, Klingenberg and McIntyre (1998) found high concordance between inter- and intraindividual variation in fly wings. However, Debat and co-workers (2000) analysing mouse craniums using a similar methodology found just the opposite. This seems to suggest that the mechanisms that affect canalization and developmental stability are related in some developmental contexts but not in others.

In organisms exhibiting symmetrical structures these issues can be studied and developmental noise can be detected and measured by the degree of asymmetry presented. Bilateral organisms can express three types of asymmetry based on the distribution of signed asymmetry values in the population: fluctuating asymmetry (FA), directional asymmetry (DA) and antisymmetry (AS) (Van Valen, 1962). In traits showing fluctuating asymmetry, the differences between left-right sides are normally distributed around a mean of zero. Directional asymmetry is characterized by a normal distribution with a mean different from zero, i.e. one side is consistently bigger. In a trait manifesting antisymmetry the differences between sides present a bimodal distribution with a mean of zero. That is: in some individuals the left side is bigger but in the others it is the right side that is bigger, the population average for the differences between both sides being zero. It is assumed that DA and AS have an adaptive basis, the left-right asymmetry being the norm in these cases and not the result of developmental problems. Consequently, these two types of asymmetry should not be used as estimators

of developmental instability (e.g. Klingenberg, 2003; Palmer and Strobeck, 2003; but see Graham *et al.*, 1998 for a different view).

Fluctuating asymmetry (FA) is broadly defined as intraindividual variation due to random differences between left and right sides. The degree of deviation from perfect bilateral symmetry reflects the balance between two opposing processes: developmental noise and developmental stability. Since the two sides of an organism result from the expression of the same genes, it is assumed that fluctuating asymmetry results from the inability of developmental programs to resist environmental perturbations. Thus, FA is often assumed to be negatively correlated with developmental stability and fitness (Moller, 1993; Palmer, 1994) and is considered a suitable estimator of developmental instability.

The genetic basis underlying developmental stability is not completely understood (e.g. Leary and Allendorf, 1989; Markow, 1995). Two possible mechanisms are genomic coadaptation and heterozygosity. In general, it is expected that increased heterozygosity decreases developmental instability whereas the breakdown in genomic coadaptation should have the opposite result (Clarke, 1993). According with Mitton and Grant (1984) the lower FA in heterozygotic individuals is probably due to dominance, over-dominance or particular gene combinations.

Interspecific hybrids are an interesting material for studies in this area. Despite the inverse relationship between the degree of fluctuating asymmetry and the percentage of protein heterozygosity, defended by some (see above), hybrids generally present greater fluctuating asymmetry than members of parental populations (e.g. Palmer and Strobeck, 1986). According with Leary and Allendorf (1989), the degree of developmental stability expressed in hybrids, results from the balance between the stabilizing effect of increased heterozygosity and the disruptive effect of the break-up of gene interactions. This balance is affected by the genetic divergence between the parental species, higher divergence levels increasing the chance for hybrids to be developmentally instable (e.g. Vrijenhoek and Lerman, 1982).

In spite of the evidences of increased fluctuating asymmetry in hybrids, the detrimental effect of hybridization on developmental stability is controversial (e.g. Markow, 1995; Alibert and Auffray, 2003). This controversy could be related with difficulties in properly estimating developmental noise (e.g. Markow, 1995; Pélabon *et al.*, 2004), and also with the traits studied.

Insect wings are a good material to use in fluctuating asymmetry studies, because they are a fitness-related trait, and their vein patterns provide landmarks easily recognizable allowing the use of geometric morphometrics. Geometric morphometrics is a powerful tool to use in fluctuating asymmetry, because it permits not only the analysis of size but also a more detailed analysis of shape (Zeldich *et al.*, 2004). Landmarks are developmental homologous points in 2D or 3D space, which do not change their position relative to other landmarks, provide coverage of the morphological trait in analysis and can be found repeatedly and reliably (e.g. Zeldich *et al.*, 2004). Geometric morphometrics involves four stages: a) recording the landmarks YX coordinates, generally two sets of coordinates are recorded for each landmark, to allow an estimate of measurement error; b) aligning the different landmark configurations using a least-squares Procrustes superimposition method (the sets of landmarks being first centered in their respective centroids and rotated to minimize the square deviations of all landmarks from their respective means); c) testing if differences in asymmetry are not due to measurement error; and finally d) testing for differences in FA among individuals or groups of individuals, e.g. different species (Palmer and Strobeck, 2003; Zeldich *et al.*, 2004).

Left-right asymmetries can be calculated using a conventional two-way mixed ANOVA analysis with side (left, right) as fixed factor and individual as random factor (e.g. Palmer and Strobeck, 1986), applied in this case to the landmark coordinates of each wing. In this model the significance of directional asymmetry is given by the side effect, while the interaction term "side x individual" is an estimate of fluctuating asymmetry where measurement error has been factored out (Palmer and Strobeck, 1986, 2003).

In any analysis using fluctuating asymmetry as a proxy for developmental instability, potential biasing factors have to be taken in consideration. For instance, body size effects can bias FA estimates. This can be corrected Ln-transforming all data, thus removing linear size dependence for FA (Palmer and Strobeck, 2003). The presence of directional asymmetry can also confound FA estimates, so ideally these analyses should avoid traits exhibiting significant DA (Palmer and Strobeck, 2003). Furthermore, even traits that exhibit "ideal" FA can express genetic variation for DA, which can influence FA estimates. This leads to the need to correct for this effect (e.g. Leamy *et al.*, 1997; Santos, 2002; Palmer and Strobeck, 2003).

One way to try to properly estimate developmental noise and remove possible bias due to genetic variation in DA, is to rely on quantitative genetic analyses devised to partition phenotypic variation into genetic and environmental components (Lynch and Walsh, 1998). This allows the further partition of the traditional estimator of FA (the interaction term "side x individual"), and determine if there is significant genetic variation for DA.

Sib analysis is a powerful tool to partition the phenotypic variance into within- and among-family components both of which can be interpreted in terms of covariances between relatives. They can also be related to the underlying causal components of variance. There are three possible designs of sib analysis: half sib, full sib, and the third being a combination of the first two (Lynch and Walsh, 1998). A "mixed" sib analysis involves the formation of harems, i.e. each male (sire) is mated with several females (dams) and several offspring of each female are analysed. Offspring of the same dam are full sibs, while the progeny of the females mated with the same male are half sibs.

An analysis of variance is then performed allowing the partition of the total phenotypic variance into the sum of the variances from each of the contributing factors. The use of paternal half-sib families allows the estimation of the additive variance component and is the best way to minimize common environmental effects and eliminate common maternal effects (Lynch and Walsh, 1998). A mixed setup using both half and full sibs provides information on the relative significance of the components of variance associated with additive and dominance effects. In this analysis we have to assume that the individuals used are random members of the same population, and that the variance component associated with epistasis is not significant.

In this case a mixed nested ANOVA with three factors is used. With this design we have: a between sire component, given by the differences between the progeny of different males; a between dam, within sire component, translated by differences between the progeny of females mated with the same male; and a within progeny component, i.e. differences between individual offspring of the same female. The use of a "mixed" sib analysis (with both half-sib and full sib breeding design) allows the partition of the terms individual and interaction sides x individual (the traditional way to estimate FA), into sire, dam and within progeny components. This design provides a more unbiased estimation of FA when significant DA is detected. In such a design, the sire and dam components are estimators of the genetic variance associated with

directional asymmetry, and provide a way to properly correct FA estimates for these effects extracted from the within progeny component. This will be a better approach to analyse the importance of developmental noise, minimizing confounding genetic effects that otherwise could not be taken into account **(see below and chapter 4).**

1.5. Adaptive potential

It is not the strongest of the species that survives, nor the most intelligent, but the one most responsive to change. Charles Darwin

Adaptation plays a fundamental role in Evolutionary Biology, first of all because of its role in shaping the temporal changes within populations. But it has also a most relevant role in defining the degree of differentiation among populations. It can result in divergence between lineages, e.g. due to evolution in different environments, ultimately giving rise to new species. However, it can also lead to convergence when different species/populations are introduced in the same environment and undergo similar selective pressures during several generations. Matos and co-workers (Matos *et al.*, 2000, 2002; Simões *et al.*, 2007a, b) presented several intraspecific studies on the evolutionary dynamics of *Drosophila subobscura* populations as they adapt to a new, laboratorial environment. These studies indicate that populations adapt to the new controlled environment. Furthermore, the authors also found indications of convergence between populations, with some repeatability between independent foundations. It will be interesting to analyse whether different species will also show convergence during laboratory adaptation. Will they converge in terms of fitness related traits, diverge or remain equally differentiated? The simplest expectation when different populations or species adapt to the same environment is convergence of fitness related traits (Futuyma, 1998). However, the specific genetic backgrounds may have a say in this, leading to different outcomes (Cohan, 1984a, b; Cohan and Hoffmann, 1989), including inability to adapt to new conditions. In fact, even the intraspecific studies of Matos and co-workers found clear differences in the evolutionary rate between independent foundations, suggesting that genetic backgrounds affect the adaptive responses (Matos *et al.*, 2002; Simões *et al.*, 2007a, b).

In addition to the obvious importance in terms of Evolutionary Biology, understanding how different species adapt to new controlled environments can also be important in terms of conservation efforts. Besides the evident relevance in terms of biodiversity, differentiated species may also present different evolutionary dynamics when faced with environmental changes i.e. they may differ in adaptive potential (e.g. England *et al.*, 2003; Reed *et al.*, 2003). In the last centuries human activities have introduced additional selective pressures, including, among others, global warming and habitat destruction and fragmentation. Furthermore, the improvements in transportation means accomplished during the last century, also introduced new ways in which colonization of new habitats can be facilitated. One such example is the recent colonization of the American continent by *Drosophila subobscura* (Brncic *et al*., 1981; Beckenbach and Prevosti, 1986). Once a typical Palaearctic species, nowadays this species can be found both in the North and South American continents where its range expanded rapidly (Gilchrish *et al.*, 2004). One possible consequence of these "man-facilitated" colonizations could be the displacement of native species. In fact, this might be the case in North America where invading *D. subobscura* could be responsible for the displacement of native *D. persimilis* (Noor, 1998).

The changes induced by man have endangered many plant and animal species, leading to the need to implement conservation measures. Among these, captive breeding plays an essential role in the conservation of many endangered species (e.g. Ralls and Ballou, 1986). Captive breeding involves the maintenance of genetic and demographic viable populations outside the species natural habitat, with the ultimate goal of reintroducing them in the wild (e.g. Frankham *et al.*, 1986; Frankham, 2002). It is expected that its importance will increase in the future, because many more species will face the risk of extinction due to habitat destruction (e.g. Soulé *et al.*, 1986; Tudge, 1995).

The lack of suitable habitats for reintroduction presents a serious problem for many endangered species, and leads to the need to maintain populations under captivity for long periods (Gilligan and Frankham, 2003). This in turn raises another problem: the evolutionary changes associated with captivity and their consequences for reintroduction. Captive populations are usually small, which may result in some problems like inbreeding, loss of genetic variation and mutation accumulation (Frankham, 2002; reviewed in Frankham, 2005a). Moreover, captivity over several

generations involves adaptation to the new environment and consequently leads to genetic changes maximizing fitness in this environment. Some of these changes could reduce fitness once these populations are released in their natural habitats, decreasing the chances of a successful reintroduction (e.g. Woodworth *et al.*, 2002). Nevertheless, the best management procedure to minimize these problems is not a consensual issue.

The problems associated with the genetic and evolutionary changes of captive populations can not be addressed studying endangered species, due both to the risks involved, as well as other obvious practical reasons (e.g. long generation times), so instead model organisms like drosophila are used (e.g. Woodworth *et al.*, 2002; Gilligan and Frankham, 2003).

The diversity of the species facing or in risk of extinction in the near future, leads us to the next problem: will different species adapt in the same manner to captivity? This is particularly important given that many endangered species are endemic or specialized in particular resources. Species with wide distributions usually are generalist species able to explore a variety of resources, and consequently have a greater potential to adapt to new conditions, namely captivity (Parsons, 1982). On the other hand, species with more restricted distributions could be expected to have a lower genetic variation (Lienert *et al.*, 2002) and consequently a lower adaptive potential (Frankham, 1995, 2005b).

In order to properly understand the mechanisms involved in adaptation to captivity and to improve conservation efforts, we need data on more species, and studies involving more than one species would be particularly interesting. It would be very important to test differences in evolutionary dynamics at the interspecific level especially in closely related species such as *D. madeirensis* and *D. subobscura* **(see below and chapter 5**).

1.6. The study system: *Drosophila madeirensis - Drosophila subobcura*

Drosophila madeirensis Monclús, 1984 is an endemic species from Madeira Island associated with Laurisilva forest (Monclús, 1984). Its close relative, *Drosophila subobscura* Collin, 1936 is a Palaearctic species, with a wide distribution including Europe, Northern Africa, and Asia Minor, being also present in the Macaronesian archipelagos of Azores, Madeira and Canary Islands (Krimbas, 1993). Moreover, in the last two decades this species successfully colonized the South and North American continents (Brncic *et al*., 1981; Beckenbach and Prevosti, 1986).

Drosophila madeirensis and *D. subobscura* can both be found in Madeira, a small volcanic island (700km^2) originated 5-6 Myr ago (Galopim de Carvalho and Brandão, 1991). This island presents the largest surviving area of Laurisilva forest, a habitat which in the Tertiary Era covered much of Southern Europe, but nowadays is restricted to some areas in Madeira, Azores, Canaries and Northen Africa. Due to its uniqueness and outstanding natural value, Madeiran Laurisilva was considered a special area of conservation under the EU Habitats Directive, with 38 named threatened plant and animal species (IUCN, 1999). Like many other islands, Madeira presents a rich fauna and flora with high levels of endemism. About 10% of the plants found in this island are endemic (Press and Short, 1994), and the terrestrial fauna presents even higher values: 15% for Diptera (Baez, 1993), and 88% for land snails (Groombridge, 1992). Laurisilva forest is being destroyed and fragmented as a result of human activities, fact that is endangering several animal and plant species, particularly endemic ones like *D. madeirensis*.

D. madeirensis and *D. subobscura* are morphologically very similar and coexist in sympatry on Madeira Island (Monclús, 1984). The analysis of nucleotide divergence at the *rp49* gene region indicates that both species have diverged rather recently, $0.6-1$ Myr ago (Ramos-Onsins *et al.*, 1998). The chromosome arrangements present in Madeiran populations of each species and the differentiation at the *rp49* gene region, suggest that the most probable scenario for the divergence between these species involves two independent colonizations from continental ancestral *D. subobscura* populations. The first originated *D. madeirensis*, which maintains a chromosome arrangement, O_3, no longer present in extant *D. subobscura* populations. Later, after the origin of another gene arrangement O_{3+4} in continental *D. subobscura*, a second

colonization originated the existing insular *D. subobscura* (Khadem *et al.*, 1998). *D. madeirensis* and *D. subobscura* present similar nucleotide variance at the *rp49* gene region, indicating that a strong founder event is not involved in the origin of *D. madeirensis* (Khadem *et al.*, 2001).

In spite of the topographical characteristics of Madeira, with deep valleys surrounded by high mountains, molecular data suggest that both *D. madeirensis* and Madeiran *D. subobscura* are each represented by a single, only slightly subdivided population (Lepetit *et al.*, 2002). The analysis of chromosome arrangements indicates a high homology between the two species (Krimbas and Loukas, 1984; Papaceit and Prevosti, 1991), the X-chromosome being the only one that underwent structural variation during the speciation process (Papaceit and Prevosti, 1989, 1991). Moreover, *D. madeirensis* chromosomes look thicker than *D. subobscura* ones and are more fragile as they break more frequently at different points (Papaceit and Prevosti, 1991).

The reproductive isolation between these species is not complete, as viable hybrids can be obtained, especially if the cross involves *D. madeirensis* females (Krimbas and Loukas, 1984; Khadem and Krimbas, 1991, 1993, 1997; Papaceit *et al.*, 1991). The cross direction involving *D. subobscura* females is harder to obtain and in general produces male-biased progeny (Khadem and Krimbas, 1991, 1993). According with several authors, e.g. Khadem and Krimbas (1993, 1997), Papaceit *et al*. (1991), hybrid males from both cross directions are sterile but hybrid females are partially fertile. However, the present work provides ample evidence that this is not always the case, and that some viable and fertile F1 hybrids from both sexes can be produced in both cross directions, particularly in the cross direction involving *D. madeirensis* females (see chapter 2). Furthermore, in our lab we were able to obtain viable and fertile hybrids until the 7^{th} hybrid generation from the more productive cross direction (C. Rego, unpublished results).

F_1 hybrids between these species present some morphological abnormalities like extra sex combs in hybrid males and abnormal head shape (e.g. Khadem and Krimbas, 1991, 1993, 1997; Papaceit *et al.*, 1991). In both abnormalities the X chromosome plays a preponderant role (Khadem and Krimbas, 1991, 1993, 1997). The genetic basis underlying these abnormalities is similar to other hybrid incompatibilities in this species pair such as hybrid sterility and inviability (Khadem and Krimbas, 1991, 1997).

Apparently *D. madeirensis* is monomorphic for chromosomal inversions (Khadem and Krimbas, 1993) a possible sign of low genetic variability.

A traditional approach to study speciation is analyzing the genetic pattern of species differences to infer the genetics of species formation (e.g. Coyne, 1983). These analyses are only possible using closely related species with incomplete reproductive isolation, when it is possible to produce viable and fertile hybrids of at least one sex. Furthermore, some species differences are only present in the hybrids, such as genetic incompatibilities expressed as morphological abnormalities. Consequently, the use of hybridization is a more accurate means to estimate species differences than interspecific comparisons (Papaceit *et al.*, 1991).

The fact that *D. madeirensis* and *D. subobscura* are two closely related species with incomplete reproductive isolation, able to produce viable and fertile hybrids, makes them an ideal system to study species differences and the speciation process involved in their divergence. For example the ability to produce hybrids of several generations, allows the use of line-cross analysis to determine which genetic effects, additive, dominance, epistasis etc., are important in the differentiation between these species (see chapter 2). Reproductive isolation is an essential part of speciation, so determining which reproductive barriers (pre-, postzygotic or both) are involved in species isolation is particularly important (see chapters 3 and 4). Assortative mating is an example of a prezygotic barrier that can prevent the detrimental consequences of interspecific matings. When two individuals from different species meet, this type of behavioural barrier is one of the first to act. So, closely related species are an ideal system to test for this barrier and its role in species isolation (see chapter 3). Hybrid sterility and inviability are examples of the most detrimental consequences of mating with heterospecifics. However this type of mating can have less extreme effects like developmental problems leading to morphological manifestations. Higher asymmetry in bilateral traits, due to higher developmental instability in hybrids can be one of such manifestations. The system *D. madeirensis* - *D. subobscura* provides a good opportunity to test for this effect (see chapter 4).

Besides the obvious importance to the study of evolutionary divergence and speciation, this study system can also provide valuable information on the adaptive potential of different closely related species. This is relevant to determine what happens when different species are placed under similar selective pressures (a new common

environment), specifically if they converge (the simplest expectation), diverge or remain equally differentiated. Studying the adaptive potential of these species is particularly interesting because they differ in terms of ecological requirements. *D. madeirensis* is an endemic species with a restricted distribution, specialized in the resources provided by Laurisilva forest, a threatened habitat, while *D. subobscura* is a generalist species with a wide geographic distribution (see chapter 5). This question is important in terms of Evolutionary Biology but also for conservation efforts that involve captive breeding for several generations.

1.7. Objectives

Speciation is one of the most controversial areas in Evolutionary Biology. The use of closely related species, with incomplete reproductive isolation, is a powerful tool to dissect general species differences and understand the genetic mechanisms involved in the speciation process. With this thesis I aim to understand several aspects related with the speciation process involved in the divergence between *D. madeirensis* and *D. subobscura*, two closely related *Drosophila* species. In this section I will detail the particular objectives I aim to address with this thesis.

1- Population divergence and the role that several genetic effects may play in fitness related traits are one of the most important issues in Evolutionary Biology. Closely related species that are able to interbreed and produce viable and fertile hybrids (e.g. F1, F2), are a valuable tool to use in this context. One of the aims I propose to achieve with the present thesis is to determine the relative contribution of additive and non-additive gene effects to the differentiation between *D. madeirensis* and *D. subobscura*. With this in mind I performed a line cross analysis comparing fitness traits between several generations (parental, F1 and F2 hybrids).

2- It is generally acknowledged that reproductive isolation is an important part of speciation. Several isolating barriers prevent the detrimental effects of hybridization. Some of these barriers prevent hybrid formation and act

before mating/fertilization has taken place. Among these, assortative mating (preferential mating between conspecifics), plays a very important role in preventing heterospecific mating and consequently, the costs associated with this type of mating. With this in mind, the second aim I propose to achieve with this work is to determine if assortative mating acts as pre-zygotic barrier in the isolation between *D. madeirensis* and *D. subobscura*. I also intend to analyze what are the consequences (if any) of heterospecific matings in terms of some life-history traits.

3 – Another important aspect of reproductive isolation, are barriers that act after fertilization has taken place and reduce hybrid fitness. In the more extreme cases these barriers include hybrid sterility and inviability. However, hybrids can also present other problems like morphological abnormalities and lower developmental stability. With this thesis I also aim to determine if F1 hybrids between *D. madeirensis* and *D. subobscura*, present higher fluctuating asymmetry (an indicator of lower developmental stability) and if this asymmetry reflects higher developmental noise in hybrids. This was done using a hybrid half-sib breeding design which allowed the partition of variance components in genetic and environmental effects.

4 – *Drosophila subobscura* and *D. madeirensis*, despite being sympatric and very closely related, differ in their ecological requirements, the former being a generalist species with a wide distribution while *D. madeirensis* is an endemic species associated with an endangered habitat and has a restricted distribution. This makes them particularly interesting to analyse in terms of their adaptive potential, in particular to common conditions under captivity. This study is particularly relevant not only in terms of Evolutionary Biology in general, but also due to its importance for conservation efforts, namely captive breeding. With this in mind, one of the aims of this work is to analyze if *D. madeirensis* and *D. subobscura* differ in the evolutionary dynamics of several life history traits during the adaptation to a new, common environment (laboratory).

1.8. Thesis structure

The current thesis is organized in 6 chapters. The first chapter is the General Introduction, where I give an overview of the several aspects addressed in the next chapters. The following 4 chapters correspond to four scientific papers which are either published (2), in press (1) or submitted (1). Each of these papers addresses one of the main objectives I proposed to analyse in the aims section.

The first objective is addressed in Chapter 2 where I present an analysis on the quantitative genetic differentiation between *Drosophila madeirensis* and *D. subobscura*, using line-cross analysis. This work was accepted for publication in *Genetica* and is currently in press.

Chapter 3 addresses my second objective: an analysis to determine if these two species present assortative mating, one possible prezygotic isolating barrier. In this work I also analyse the consequences of mating with heterospecific individuals in terms of life history traits, namely if there are fitness costs involved. This work has been submitted to *Behavior Genetics*.

In Chapter 4, I present a study addressing if developmental stability is lower in interspecific hybrids than in parental species, using fluctuating asymmetry as an estimator of this trait. The experimental setup used, a hybrid half-sib breeding design, allowed the dissection of several variance components to properly estimate if hybrids present higher developmental noise. This work was published in *Evolution*.

In Chapter 5, I address the last objective of this thesis, a comparison of the adaptive potential between the two study species, *D. madeirensis* and *D. subobscura*, analysing the evolutionary trajectories of several life history traits, during the adaptation to a new environment (the laboratory) throughout several generations. This work was published in *Physiological Biochemical Zoology*.

Finally, in Chapter 6, I present a general discussion of the major findings of this work, their implications for several areas of knowledge as well as future directions for further work.

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Chapter 2.

Genetic differentiation between *Drosophila madeirensis and Drosophila subobscura*

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ORIGINAL PAPER

Quantitative genetics of speciation: additive and non-additive genetic differentiation between *Drosophila madeirensis* and *Drosophila subobscura*

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Abstract The role of dominance and epistasis in population divergence has been an issue of much debate ever since the neoDarwinian synthesis. One of the best ways to dissect the several genetic components affecting the genetic architecture of populations is line cross analysis. Here we present a study comparing generation means of several life history-traits in two closely related *Drosophila* species: *Drosophila subobscura*, *D. madeirensis* as well as their F_1 and F_2 hybrids. This study aims to determine the relative contributions of additive and non-additive genetic parameters to the differentiation of life-history traits between these two species. The results indicate that both negative dominance and epistatic effects are very important in the differentiation of most traits. We end with considerations about the relevance of these findings for the understanding of the role of non-additive effects in speciation.

Keywords Speciation · Generation means · Hybrid breakdown · Dominance · Epistasis · *Drosophila madeirensis* · *Drosophila subobscura*

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Introduction

Population differentiation is a central issue in evolutionary biology. Fisher and Wright, two fundamental contributors to the neoDarwinian synthesis, disagreed on the processes underlying the evolution of natural populations. Specifically, they disagreed on the role that additive and non-additive genetic factors play in population differentiation. According to Fisher selection acts primarily on individual loci, and non-additive effects have little evolutionary importance (Fisher 1930). On the other hand, Wright's shifting balance theory of evolution relies on epistatic gene action (Wright 1977) and the formation of coadapted gene complexes is fundamental in his model (Fenster et al. 1997). In spite of the considerable theoretical and empirical developments in this area, the controversy is far from solved (e.g. Coyne et al. 1997, 2000; Wade and Goodnight, 1998; Goodnight and Wade 2000; Gravilets 2004). One of the motives is the paucity of empirical studies that test the role of epistasis in the evolution of fitness related traits (Barton and Turelli 1989; Whitlock et al. 1995; Fenster et al. 1997).

Non-additive gene action has been commonly associated with population differentiation (Lynch and Walsh 1998). Dominance effects are relatively abundant in the literature and are frequently expressed as heterosis (e.g., Bieri and Kawecki 2003; Edmands 1999; Facon et al. 2005; Fenster and Galloway 2000; but see Teotónio et al. 2004 for evidences of negative dominance). Comparatively, evidence of epistasis is scarcer, not very consistent and comes mainly from intraspecific studies (Blows and Sokolowski 1995; Starmer et al. 1998; Gilchrist and Partridge 1999; Fenster and Galloway 2000; Carrol et al. 2001, 2003; Bieri and

Kawecki 2003; Fox et al. 2004; Teotónio et al. 2004; Bradshaw et al. 2005). Intraspecific hybrids between species can express outbreeding depression or hybrid breakdown—having lower fitness than the parental species (Waser and Price 1989, 1994; Brown 1991; Burton 1990; Leberg 1993; Fenster and Galloway 2000; Templeton 1981; Coyne and Orr 1989, 1997). Hybrid breakdown is generally attributable to the disruption of favourable gene interactions that have evolved independently in the two parental types and is expected to occur in more differentiated populations or species, whether it is partly a cause or just a consequence of the reproductive isolation, as mentioned in the Dobzhansky-Muller model (see Fenster et al. 1997; Johnson 2002; Gavrilets 2004).

Line-cross analysis is a powerful way to dissect the relative contributions of additive and non-additive genetic effects to population differentiation (e.g., Mather and Jinks 1982; Lynch 1991; Lynch and Walsh 1998; Kearsey and Pooni 1996). However, to properly dissect these effects it is necessary to compare several hybrid generations (e.g., F_1, F_2 hybrids and/or backcrosses with the parentals, see Mather and Jinks 1982). The scarcity of evidences for epistasis is in part due to these demanding designs (e.g., difficulties in obtaining hybrids of more than one generation) and to a low statistical power to detect these effects (cf. Lynch and Walsh 1998). In spite of all the inherent difficulties, evidence for epistasis in studies involving different species has been found both in plants (e.g., Macnair and Cumbs 1989; Fritz et al. 2003), and animals (e.g., Breeuwer and Werren 1995; Hatfield 1997). Species that hybridize successfully for several generations are thus a valuable material to explore in these issues. Such is the case of the species pair *Drosophila madeirensis*–*Drosophila subobscura*.

Drosophila madeirensis Monclús and *D. subobscura* Collin are two closely related species that coexist on Madeira Island, the former being endemic. The estimated time of divergence between both species is 0.6–1.0 Myr ago (Ramos-Onsins et al. 1998). However, they are not completely isolated reproductively, as some crosses produce fertile hybrid females and sterile males in both directions (Khadem and Krimbas 1991, 1993, 1997; Papaceit, San Antonio and Prevosti 1991), F_1 hybrids being easier to obtain when *D. madeirensis* is the maternal species. Crossing *D. madeirensis* females with *D. subobscura* males yields progeny with a 1:1 sex ratio, but the reciprocal cross tends to be male biased (Khadem and Krimbas 1991, 1993). However, in our particular case, it was possible to produce fertile male hybrids in both directions, and F_2 progeny could be obtained, though the *D. subobscura* females–*D. madeirensis* males direction proved to be much harder, basically due to the extremely male biased sex ratio in the F_1 hybrids (Rego et al. 2006).

In this study we investigated the genetic basis of evolutionary divergence of several fecundity related traits and survival between *D. madeirensis* and *D. subobscura* by comparing the mean values of several generations: parental, F_1 and F_2 hybrids. By testing several genetic models we were able to infer which genetic effects, additive, and non-additive (dominance, epistasis and maternal) may be contributing to the differentiation between these two species.

Materials and methods

Population stocks and crosses

The *D. madeirensis* and *D. subobscura* base stocks were derived from a sample of wild flies collected at Ribeiro Frio (Madeira Island; for details see Rego et al. 2006). Laboratory populations of both species were set up in April 2001 and split into three replicates (m_1, m_2, and m_3 for *D. madeirensis*; s_1, s_2, and s_3 for *D. subobscura*) at generation 3. All replicated populations were kept on a discrete generation (of 30 days), controlled larval and adult densities regime at 18°C on a 12:12 light:dark period (see Matos et al. 2000; Matos et al. 2002). The number of breeding adults per population was typically around 1,000 flies, never dropping below 400. The assays in the present study were made after 23 generations of adaptation to laboratory conditions.

For each pair of replicated populations reciprocal F_1 hybrids were obtained by mass crossing 250 virgin females and 250 virgin males. The mass crosses ♀♀ *D. madeirensis* (m_i; $i = 1, 2, 3$) × ♂♂ *D. subobscura* (s_i) gave the series $F_1 \cdot m_1 s_1$, $F_1 \cdot m_2 s_2$, and $F_1 \cdot m_3 s_3$ (i.e., the maternal species is always indicated first); and the mass crosses ♂♂ *D. subobscura* × ♀♀ *D. madeirensis* the series $F_1 \cdot s_1 m_1$, $F_1 \cdot s_2 m_2$, and $F_1 \cdot s_3 m_3$. All $F_1 \cdot m_i s_i$ produced F_2 progeny when hybrid females and males were mass-crossed (hereafter referred to as $F_2 \cdot m_1 s_1$, $F_2 \cdot m_2 s_2$, and $F_2 \cdot m_3 s_3$, respectively); however, only the crosses involving individuals from $F_1 \cdot s_2 m_2$ produced enough F_2 hybrids (i.e., $F_2 \cdot s_2 m_2$) as to be included in the present study. The reason was that F_1 hybrids were harder to obtain when *D. subobscura* was the maternal species and, in addition, the sex ratio was greatly male biased. All generations (parental, F_1 and F_2) were assayed synchronously, which involved the formation of F_1 hybrids on two separate occasions: the first to produce the F_2 generation and the second to obtain the F_1

individuals for the assays. All fly handling was done at room temperature (22–24°C) using CO_2 anaesthesia when necessary.

Assays of fitness traits

We measured age of first reproduction, early and peak fecundity, and female survival from a total of 12 individual couples of virgin flies from each replicated parental, F_1, and F_2 populations. Each couple was placed in a vial containing 1 ml of Drosophila medium less than 4 hours after eclosion. During two weeks the flies were transferred daily to new vials and the eggs laid by each female were counted. Age of first reproduction was measured as the number of days until a female laid her first egg since emergence, early fecundity as the number of eggs laid during the first week, peak fecundity as the number of eggs laid during the second week, and survival as the number of days the female remained alive during the fecundity assays (i.e., the upper bound for survival was two weeks).

Age of first reproduction was estimated conditional to the female not dying before the first egg appeared and, therefore, we discarded a few females that died before the third day since emergence. Similarly, early fecundity was estimated conditional to the female being alive at the end of the first week, and peak fecundity conditional to being alive on the last day of the assay (day 14).

Analysis of generation means

To properly estimate several composite genetic parameters using Mather and Jinks' coefficients (1982)—specifically the several types of digenic interactions—both types of F_2 hybrids and backcrosses are needed (Mather and Jinks 1982; Kearsey and Pooni 1996; Lynch and Walsh 1998). Since we do not have data from backcrosses we only tested here for the presence of the composite additive effect $[a]$ (i.e., the sum of individual effects of loci with both alleles derived from the same parental species); the composite dominance effect $[d]$ (the sum of individual effects of loci with alleles derived from the two species); a composite epistasis effect $[e]$, which includes here the epistatic terms describing additive × additive, additive × dominance, and dominance × dominance epistatic interactions; and maternal effects $[m]$.

The first estimation is the genetic difference between the two species, obtained by comparing their means: $[a] = \bar{m}_i - \bar{s}_i; i = 1, 2, 3$. Conformity with a purely additive model means that F_1 hybrids would be at the midpoint from the parental values, which can be tested as:

$$[\Delta_d] = \left(\overline{F_1 \cdot m_i s_i} + \overline{F_1 \cdot s_i m_i}\right) - \left(\overline{m_i} + \bar{s}_i\right); i = 1, 2, 3;$$

i.e., as the difference between the average trait in F_1 hybrids to that in the parental species. Following a similar reasoning, the conformity to the additive-dominance model can be tested as:

$$[\Delta_e] = 2\left(\overline{F_2 \cdot m_i s_i} + \overline{F_2 \cdot s_i m_i}\right) - \left(\overline{F_1 \cdot m_i s_i} + \overline{F_1 \cdot s_i m_i}\right) - \left(\overline{m_i} + \bar{s}_i\right); i = 1, 2, 3.$$

Since only one $F_2 \cdot s_i m_i$ replicate was available (i.e., $F_2 \cdot s_2 m_2$), we used a slightly modified version of Δ_e to test for epistasis (see below). Finally, the difference in mean phenotypes of daughters from the two reciprocal F_1 crosses allows testing for maternal effects.

Statistical analyses were performed by means of two-way mixed ANOVAs, with generation as fixed and replicate as random factors. Statistical significance of each composite effect was tested via orthogonal contrasts between the corresponding means (each comparison or contrast has one degree of freedom). Table 1 gives the contrast coefficients we used. The generation × replicate interaction terms provided the appropriate error terms, thus avoiding the heteroscedasticity problem due to the higher within-family variance in the F_2 generation (Mather and Jinks 1982).

Results

Averages for the fitness traits assayed are plotted in Fig. 1, and statistical analyses are shown in Table 2. It is worth noting that replicated crosses performed quite similarly as non-significant differences were generally detected for the 'replicate' effect. This suggests that using only one replicate for F_2 hybrids when *D. subobscura* was the maternal species (i.e., $F_2 \cdot s_2 m_2$) does not introduce a substantial bias in the analysis.

The only fitness trait that was noticeably different between *Drosophila subobscura* and *D. madeirensis* was fecundity; with *D. subobscura* laying substantially more eggs in both fecundity periods (early fecundity: $[\hat{a}] = -39.0$; peak fecundity: $[\hat{a}] = -40.3$; caret denotes "an estimator of").

The F_1 hybrids from both cross directions only differed between them in survival ($[\hat{m}] = 1.6$), $P < 0.01$), clearly indicating maternal effects for this trait but not for fecundity-related traits. Cytoplasmic gene(s) in *D. subobscura* seem to play an important role in decreasing survival of F_1. s_i m_i hybrids (Fig. 1).

Table 1 Contrast coefficients for the four composite genetic parameters. [*a*]– additive effects, [*d*]–dominance effects, [*e*]–epistatic effects [*m*] – maternal effects

	D. madeirensis			*D. subobscura*			♀♀ *D. madeirensis* × ♂♂ *D. subobscura*			♀♀ *D. subobscura* × ♂♂ *D. madeirensis*			♀♀ $F_1 \cdot m_i s_i \times$ ♂♂ $F_1 \cdot m_i s$			♀♀ $F_1 \cdot s_2 m_2 \times$ ♂♂ $F_1 \cdot s_2 m_2$
	m_1	m_2	m_3	s_1	s_2	s_3	$F_1 \cdot m_1 s_1$	$F_1 \cdot m_2 s_2$	$F_1 \cdot m_3 s_3$	$F_1 \cdot s_1 m_1$	$F_1 \cdot s_2 m_2$	$F_1 \cdot s_3 m_3$	$F_2 \cdot m_1 s_1$	$F_2 \cdot m_2 s_2$	$F_2 \cdot m_3 s_3$	$F_2 \cdot s_2 m_2$
[*a*]	1	1	1	–1	–1	–1	0	0	0	0	0	0	0	0	0	0
[*d*]	–1	–1	–1	–1	–1	–1	1	1	1	1	1	1	0	0	0	0
[*e*]	–1	–1	–1	–1	–1	–1	–1	–1	–1	–1	–1	–1	3	3	3	3
[*m*]	0	0	0	0	0	0	1	1	1	–1	–1	–1	0	0	0	0

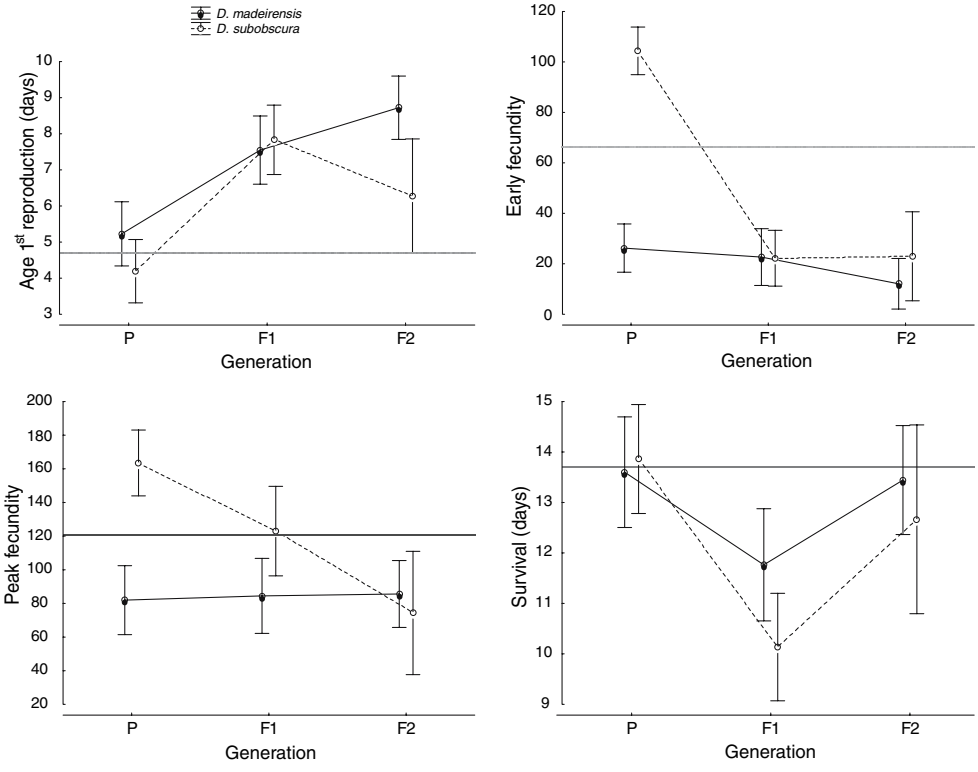

Fig. 1 Generation means for the parental species *D. madeirensis* and *D. subobscura*, and their F_1 and F_2 hybrids from both reciprocal crosses, for all analysed traits: age of first reproduction, early fecundity, peak fecundity and survival. Full dots: *D. madeirensis* and $F_1$and F_2 hybrids with this species as maternal species; empty dots: *D. subobscura* and $F_1$and F_2 hybrids with *D. subobscura* as maternal species. Lines connect the dots of the same maternal direction. Standard errors and a line indicating the mid-parent value for each trait are also given

Though F_1 hybrids had lower survival, F_2 hybrids presented similar values to the parental species.

Overall, the results indicated that F_1 hybrids performed worse than the mid-parent (age first reproduction: $\left[\hat{d}\right] = 3.0$; early fecundity: $\left[\hat{d}\right] = -42.6$; peak fecundity: $\left[\hat{d}\right] = -19.8$; survival: $\left[\hat{d}\right] = -2.8$). However, when compared to the maternal species it was clear that the significant drop in early fecundity was mainly relative to *D. subobscura* (Fig. 1) since the F_1 hybrids performed more or less alike *D. madeirensis* (i.e., dominance for fecundity was toward *D. madeirensis*): the average dominance $[\hat{d}]/[\hat{a}]$ was equal to 1.1.

Peak fecundity was the only fitness trait where a simple additive genetic model was adequate (Table 2). For all other traits epistasis was statistically significant, despite difficulties to quantify it with sample sizes as small as these here. Using the contrast coefficients for [*e*] in Table 1 to measure epistasis, the resulting values were as follows. Age of first reproduction: $[\hat{e}] = 22.8$; early fecundity: $[\hat{e}] = -348.3$; peak fecundity: $[\hat{e}] = -355.1$; survival: $[\hat{e}] = 11.0$. The figures always point in the direction of F_2 progeny being less fit than the parental species and/or F_1 hybrids (Fig. 1).

Of the several parameters tested, [*d*] was the most consistent. Dominance effects were highly significant in three of the four analysed traits (Table 2). This indicates that dominance effects may play an important role in the differentiation of life-history traits between *D. madeirensis* and *D. subobscura*. Epistatic effects [*e*] seem also to be very important, as their presence was detected in all traits with the exception of peak fecundity.

Table 2 ANOVAs for the fitness traits assayed (age at first reproduction, early fecundity, peak fecundity, and survival) measured for six generations (parental species *D. madeirensis* and *D. subobscura*, two F_1 hybrids, and two F_2 hybrids) with up to three replicated populations each. Composite genetic parameters were tested from orthogonal linear contrasts (see Table 1). The denominator used to calculate F-values for main effects and contrasts is the corresponding replicate × generation interaction

	Source of variation	df	SS	MS	F
Age of first reproduction	Replicate(R)	2	29.4	14.7	1.24
	Generation(G)	5	509.6	101.9	8.56**
	[*a*]	1	18.8	18.8	1.58
	[*d*]	1	292.2	292.2	24.50**
	[*e*]	1	125.0	125.0	10.49*
	[*m*]	1	0.1	0.1	0.01
	R × G	8	95.4	11.9	1.76§
	Error	163	1106.5	6.8	
Early fec.	Replicate	2	648.0	324.0	0.20
	Generation (G)	5	201672.5	40334.5	24.44***
	[*a*]	1	108073.2	108073.2	65.57***
	[*d*]	1	55835.5	55835.5	33.88***
	[*e*]	1	27738.8	27738.8	16.83**
	[*m*]	1	11.7	11.7	0.01
	R × G	8	13185.5	1648.2	1.95§
	Error	155	139079.7	799.3	
Peak fec.	Replicate	2	9403.7	4701.8	0.57
	Generation	5	179479.1	35895.8	4.26*
	[*a*]	1	108333.3	108333.3	12.86**
	[*d*]	1	10438.3	10438.3	1.24
	[*e*]	1	27181.9	27181.9	3.23
	[*m*]	1	15975.5	15975.5	1.90
	R × G	8	67372.3	8421.5	2.71**
	Error	141	621682.1	3814.0	
Survival	Replicate	2	20.1	10.0	3.54§
	Generation(G)	5	371.2	74.2	26.38***
	[*a*]	1	1.2	1.2	0.42
	[*d*]	1	276.9	276.9	97.95***
	[*e*]	1	30.1	30.1	10.63*
	[*m*]	1	46.0	46.0	16.26**
	R × G	8	22.6	2.8	0.25
	Error	174	1785.6	11.0	

Note: Analyses were carried out in STATISTICA V6, with Type III sums of squares.

§ $0.10 > P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Discussion

Genetic differentiation between *Drosophila subobscura* and *Drosophila madeirensis*

There is a clear genetic differentiation in life-history traits between *Drosophila subobscura* and *Drosophila madeirensis*. This differentiation involves additive and non-additive effects, the latter appearing in most traits analysed. Both dominance and epistasis are involved, and outbreeding depression is expressed since the first hybrid generation.

Drosophila subobscura generally showed a higher performance compared to *Drosophila madeirensis* for all life history traits. This difference was highly significant for early and peak fecundity, though not for age of first reproduction and survival. However, our failure to detect significant differences between the two species in the last two traits does not mean that they are not genetically different. In fact the detection of significant dominance effects for both traits suggests that the parental species are in fact genetically different, because dominance is strongly affected by the heterozygosity of the genes for which the species differ (Kearsey and Pooni 1996).

Epistatic interactions were also frequently involved. Peak fecundity was the only trait where epistasis was not detected. A misleading effect can come from the presence of maternal effects, which can be confounded with epistasis (Kearsey and Pooni 1996). However, in our particular case, survival was the only trait where maternal effects were detected, which renders unlikely that our general finding of epistasis are only due to these effects.

For both fecundity traits, the estimated [*e*] and [*d*] values were negative. This suggests that both dominance effects between the two species and dis-

ruption of gene combinations within each species lead to a reduction of fitness in hybrids. Survival also presented a drop of performance in F_1 hybrids, corresponding to a negative [*d*], though, somewhat surprisingly F_2 hybrids presented an improvement, getting close to the mean parental values, corresponding to a positive [*e*].

Though we have been interpreting [*d*] and [*e*] values as indicating dominance and epistasis, respectively, the actual scenario is a bit more complicated than that. According with Kearsey and Pooni (1996), [*d*] is affected by several genetic parameters, of which only one is dominance. Specifically in our estimates [*d*] = 2[*D*] + 2[*DD*] (considering maternal effects irrelevant), where *D* stands for dominance and *DD* for dominance-by-dominance digenic composite effects. Similarly, [*e*] is equal to –2[*aa*]– [*DD*] (in the absence of maternal effects), where [*aa*] stands for additive-by-additive composite effects.

By comparing these two parameters we can try to infer the particular importance of the several genetic effects involved. If only dominance-by-dominance composite effects were involved, we would expect [*e*] to be similar to [*d*]/2. None of the comparisons suggests such a simple scenario. In fact, [*d*]/2 was smaller than [*e*] in absolute values and of the same sign for early fecundity and age of first reproduction. This, together with the values presented by the several generations (see Fig. 1) does not allow us to exclude any of the potential contributions of dominance and of the two epistatic effects. As for survival, combining the information of [e] (positive), [d] (negative) and Fig. 1 suggests the presence of dominance and digenic dominance-by-dominance epistasis (since F_2 is close to the parentals, not expected by additive epistasis). In this particular case a more complex model including additive epistasis is not needed.

Comparisons with other studies

Evidence for epistasis by means of line cross analysis are relatively scarce in the literature, both due to the demanding designs and low statistical power (see Lynch and Walsh 1998). Nevertheless, some indications of epistatic effects have been obtained with this method (e.g., Macnair and Cumbs 1989; Breeuwer and Werren 1995; Hatfield 1997; Starmer et al. 1998; Fritz et al. 2003). Other methodologies applied to studies on population differentiation look promising to test for epistasis, and non-additive effects in general, such as QTL analysis (e.g. Li et al. 1997a, b; Orr and Irving 2001). Though general methodological difficulties also applies to QTL analysis (Tanksley 1993; Orr 2001), recent developments in this area have improved the ability to detect these effects (e.g. Baierl et al. 2006; Blanc et al. 2006).

Line cross analysis in intraspecific crosses are more abundant and give contrasting results in the genetic effects detected, both between and within studies (e.g., Edmands 1999; Bieri and Kawecki 2003; Teotónio et al. 2004). Teotónio et al. (2004), studying highly differentiated *D. melanogaster* populations, found little evidence of epistasis. These authors compared two selective regimes with their respective controls, one regime selected for increased starvation resistance and the other for accelerated development. They found that the only trait that revealed epistasis was male starvation resistance, curiously in the regime selecting for accelerated development, less differentiated for starvation resistance. On the other hand, our interspecific study presents several suggestions of epistatic effects, both in fecundity related traits and survival. The discrepancy in finding epistatic effects, both between studies and traits, could be generally related with the degree of differentiation presented by the populations in each trait, particularly considering studies involving populations from the same species (Edmands 1999; Bieri and Kawecki 2003; Teotónio et al. 2004) vs. the interspecific analysis in our case. Nevertheless, there is no simple rule, as the study of Teotónio et al. (2004) illustrates. Lair et al. (1997) suggested that additive effects may be more important in the early stages of divergence, whereas differences due to epistasis arise after longer periods of isolation. However, differentiation due to epistatic effects can arise very quickly (100 generations) during population divergence (e.g. Carrol et al. 2001, 2003; but see Teotónio et al. 2004 for contrasting results). It seems thus that there is no simple rule allowing generalizations from the results. There is also some evidence that the genetic basis of differentiation may vary according to the trait analysed (e.g., Crnokrak and Roff 1995; Orr 2001; Carroll et al. 2003). For instance, Orr (2001) in a review of studies on the genetics of species differences found that hybrid sterility and inviability involve more frequently epistasis and recessivity than other species differences. These several factors may explain discrepancies of results among studies.

Does non-additivity play a role in speciation and maintenance of specific diversity?

The presence of negative dominance and epistasis effects in the differentiation of our species, does not allow us to infer that these interactions were a cause of speciation (Coyne 1992; Fenster et al. 1997). Accord-

ing to the Dobzhansky-Muller model maladaptive genotypes only appear in the hybrids of well differentiated populations and not in the ancestral populations, previous to genetic differentiation. If this is the case, then epistasis will not promote, at least directly, evolutionary divergence, as defended in a Wrightanian scenario; it will only be a consequence of this process (see Fenster et al. 1997; Johnson 2002). The same reasoning can be applied to negative dominance effects as the ones also obtained in this study. Having said this, the finding of negative epistasis and dominance in the differentiation between species is relevant for the discussions about the role of such genetic effects on speciation. There is now growing evidence that gene interaction may play an important role in speciation (e.g. Aspi 2000; Wade 2002). It is likely that epistasis is also responsible, at least in part, for fostering the evolution of mechanisms causing reproductive isolation, preventing the formation of maladapted gene combinations in the hybrids (Whitlock et al. 1995; Turelli and Orr 2000; Orr 2001, Wade 2002). Negative dominance, as we found in this work, may lead to similar evolutionary scenarios. Curiously, the literature focus much more on epistasis (see Orr 2001).

The major finding of our work is the detection of significant negative dominance and epistatic effects, contributing to the differentiation in life history traits between *Drosophila madeirensis* and *Drosophila subobscura*. This type of genetic differentiation may have contributed to the speciation event per se and/or to the reinforcement of genetic and evolutionary barriers that maintain these species. As more and more empirical data appear similar to ours, we will hopefully be able to answer the ultimate question: what is a cause and what is a consequence of the speciation event? For now, speciation remains ''the mystery of mysteries'' as Darwin had already called it.

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Chapter 3.

Interspecific mating discrimination in *Drosophila* and its effects on life history traits

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Interspecific mating discrimination in *Drosophila* and its effects on life history traits

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Abstract

Assortative mating is one of the most important pre-zygotic barriers preventing gene flow between species. In many species reproductive isolation is incomplete; with interspecific crosses showing mating asymmetry. With this study we aim to determine whether *Drosophila madeirensis* and *D. subobscura*, two closely related species, show assortative mating, whether there is mating asymmetry and whether interspecific matings affect life history traits. We present data indicating assortative mating in these species, the heterospecific cross involving *D. subobscura* females being easier and more fecund. However, previous studies indicate that this cross gives fewer hybrids, mainly males, while the reciprocal cross yields more progeny with an even sex-ratio. Altogether these data suggest that different reproductive barriers are involved in the two cross directions between *D. madeirensis* and *D. subobscura*. Implications for speciation processes are discussed.

Keywords: *Drosophila madeirensis, Drosophila subobscura*, mating behavior, interspecific mating, fecundity, speciation

Introduction

Speciation involves the evolution of reproductive isolation preventing hybridization and gene flow between populations. Reproductive barriers are usually divided in two categories: prezygotic barriers, which prevent the formation of hybrids, and postzygotic barriers which reduce hybrid fitness, preventing gene flow after mating/fertilization has taken place (e.g. Futuyma 1998; Gavrilets 2004).

There is some indication that prezygotic and postzygotic barriers evolve at different rates, namely with sympatric pairs of populations developing assortative mating faster than hybrid incompatibilities (e.g. Coyne and Orr 1989; 1997; Mallet 2006).

Positive assortative mating is an example of a prezygotic barrier and happens when individuals discriminate between potential mating partners, choosing individuals from their own population/species. Differences in courtship behavior can lead to individuals discriminating between potential sexual partners (e.g. Kaneshiro 1976; Ewing 1983; Spieth and Ringo 1983). This discrimination can involve an intricate signal exchange between sexes, the signals being chemical, visual, acoustic or a combination of some of them (e.g. in *Drosophila* see Ewing 1983; Spieth and Ringo 1983; Hoikkala 1988). This mutual exchange of signals allows the recognition of suitable mating partners, preventing the detrimental effects of producing maladapted hybrids.

However, the degree of discrimination in mate choice depends on the costs associated with mating for each sex, the sex with the higher costs is expected to be more discriminating. In general females are more discriminating, as they usually have higher reproductive investment (e.g. Andersson 1994; Shuster and Wade 2003). Mating with an individual from another species can have significant fitness costs for females, such as reduced oviposition rate, life-time fecundity and longevity (Wade et al. 1994; Sota and Kubota 1998, Shapiro 2000), as well as the production of maladapted hybrids. In many cases hybrids between species are sterile (at least one of the sexes) or, in more extreme cases, inviable, which has led to the traditional view that hybrids are evolutionary "dead ends" (Mayr 1963, but see Arnold 1997).

Although hybrid unfitness and reproductive isolation are very important aspects of population differentiation, it is unlikely that genes causing hybrid unfitness or

reproductive isolation are responsible for speciation. In fact, it is probable that most hybrid unfitness arose after the speciation event (Mallet 2006). Actually many closely related sympatric species show strong isolation but little hybrid unfitness in laboratorial trials (Mallet 2006). Thus the ecological view of speciation is that reproductive isolation is not directly selected, but it is a side-effect of divergent selection on other traits (e.g. Rice and Salt 1990; Schluter 2001). Evidence for this type of speciation has been growing in recent years (e.g. Bush and Butlin 2004; Rundle and Schluter 2004; Waser and Campbell 2004; Duran and Rützler 2006; Nosil and Crespi 2006). There is also increasing evidence that reproductive isolation among many species is incomplete (e.g. Mallet 2006), and that hybridization and introgression are frequent both in animals and plants (Grant and Grant 1992; Arnold and Hodges 1995; Wang et al. 1997; Mallet 2005). Hybridization has even been known to "promote" speciation, originating new species, especially in plants (e.g. Grant 1966; Rieseberg 1997; Pires et al. 2004; for an example in fish see Alves et al. 2001; for butterflies see Gompert et al. 2006 and for fungi see Ioos et al. 2006) and is considered to be an important evolutionary mechanism (Barton 2001).

Many *Drosophila* species present asymmetric reproductive isolation, meaning that interspecific crosses in one direction are easier than in the other (Ödeen and Florin 2002). This asymmetry has even been considered an indicator of the direction of evolution (Kaneshiro 1976; 1980; Watanabe and Kawanishi 1979). Kaneshiro's hypothesis states that the females from the "ancestral" species are more discriminating against the males from the "derived" species than "derived" females are relative to "ancestral" males (Kaneshiro 1976; 1980). Watanabe and Kawanishi (1979), defend the opposite: namely that "derived" females are the ones which are more discriminating. These hypotheses have been amply debated (e.g. Markow 1981; Moodie 1982; Fraser and Boake 1997; Ödeen and Florin 2002).

The literature on reproductive isolation has focused chiefly on behavioral traits involved in assortative mating, acting as pre-zygotic barriers, and on hybrid inviability and/or sterility as post-zygotic barriers (e.g. Coyne and Orr 2004). However, reproductive isolation, or at least limited gene flow, may also occur due to the lower fecundity of females mated heterospecifically, acting as a postmating, probably prezygotic, reproductive barrier. Examples of lower fecundity in heterospecific matings have been found in several species (e.g. Collins and Margolies 1991; Wade et al. 1994; Sota and Kubota 1998; Shapiro 2000). In spite of the ease with which many *Drosophila*

species can be maintained in the laboratory, there are not many studies addressing this issue in *Drosophila*. One exception is Price et al. (2001), who analyzed the consequences of heterospecific matings in female life history traits in the *Drosophila simulans* species complex.

Drosophila madeirensis Monclús and *D. subobscura* Collin are two closely related *Drosophila* species with incomplete reproductive isolation. They are morphologically rather similar, coexisting in sympatry on Madeira Island, *D. madeirensis* being an endemic species associated with Laurisilva (Monclús 1984). The estimated time of divergence for these species is 0.6 to 1 Myr (Ramos-Onsins et al. 1998). *D. madeirensis* is thought to be derived from a colonization by continental *D. subobscura* while extant Madeiran *D. subobscura* most probably derived from an independent colonization event (Khadem et al. 1998). F1 hybrids between these species are relatively easy to obtain under laboratory conditions, especially if the mother species is *D. madeirensis* (Khadem and Krimbas 1991; 1993; Papaceit et al. 1991; Rego et al. 2006; 2007b), and there is some indication that hybrids can also occur under natural conditions (Khadem et al. 2001).

D. subobscura courtship was studied by several authors (e.g. Wallace and Dobzhansky 1946; Spieth 1952; Maynard Smith 1956; Steele 1986a, b). Courtship in this species consists mostly of visual signs exchanged by both sexes, involving an intricate dance performed by the males (Maynard Smith 1956), the presence of light being essential (e.g. Wallace and Dobzhansky 1946). In contrast with the data on *Drosophila subobscura*, there are no published studies on *D. madeirensis* mating behavior.

Here we compare mating behavior and life history traits of *D. madeirensis* and *D. subobscura* mated with conspecific and heterospecific individuals. Some data on life history traits of F1 hybrids are also given. We aim to address the following questions:

Is there assortative mating in these species?

Do these species differ in the tendency to mate with conspecifics *versus* heterospecifics?

Are there fitness costs of heterospecific matings, specifically do life history traits differ between conspecific and heterospecific matings?

Do hybrids present a lower performance than the parental species?

Materials and Methods

Population stocks and maintenance

In 2001 laboratory populations of *D. madeirensis* and *D. subobscura* were founded using wild flies collected in Ribeiro Frio, Madeira Island. In the third generation these populations were split in three independent replicate populations (m_i for *D. madeirensis* and s_i for *D. subobscura*, i =1,2,3). The flies were kept in controlled conditions in an incubator at 18ºC with a photoperiod of 12L/12D. The maintenance regime involved discrete generations (30 days) with controlled adult (50 individuals per vial) and larval (70-80 eggs per vial) densities (see Matos et al. 2000; 2002; Rego et al. 2006; 2007a, b for details).

Mating behavior assays

Assays on mating behavior were carried out when the populations were in their sixth generation after foundation from the wild. Virgin individuals were collected and sexed within 6-8 hours after emergence and they were placed in vials in groups of 10 individuals of the same sex, until the time they were assayed (7-9 days of age). *D. subobscura* reaches sexual maturity with 8 days of age (Monclús and Prevosti 1971)

Before each assay the flies were kept in absolute darkness for 12 hours to stimulate mating behavior. No-choice mating experiments were used, placing one male and one female in each observation vial without CO_2 anaesthesia. All four possible mating combinations were assayed synchronously: conspecific (m - *D. madeirensis*, s - *D. subobscura*) and heterospecific (ms - ♀ *D. madeirensis* x ♂ *D. subobscura* and sm - ♀ *D. subobscura* x ♂ *D. madeirensis*). Each observation period comprised 12 mated pairs, three of each mating type (m, s, sm and ms), pairing populations in each observation period according with replicate number (e.g. m_1, s_1, m_1s_1, s_1m_1), so that in each block of observations only one replicate of each species was represented. The observation period lasted 45 minutes and the following parameters were recorded: CL- courtship latency (time elapsed between placing the flies together and the beginning of courtship), a trait which indicates how long the male takes to identify a female as a potentially receptive mate; CD- courtship duration (time elapsed between courtship beginning and mating), a measure of female receptivity; ML – mating latency (time

elapsed between the individuals being placed together and the occurrence of mating); and MD - mating duration (time elapsed between the beginning and ending of mating). All time measurements were taken in seconds. The number of courtships and matings was also registered. Around 24 mated pairs were assayed per population for each mating type.

Fecundity assays

Comparing species

Fecundity was assayed with pairs (at generations 7 after foundation from the wild) and with groups (at generation 11). In the assay with pairs, conspecific (m, s) and heterospecific (ms, sm) mated pairs of both species were placed individually in vials containing culture medium. The three replicate populations of each species were used, pairing individuals according with replicate number (e.g. m_1, s_1, m_1s_1, s_1m_1). The mated pairs were formed using virgin individuals which were sexed using CO_2 anaesthesia less than 6 hours after adult eclosion. The mated pairs were transferred daily to new vials and the eggs were counted. In these assays, the traits analyzed were age of first reproduction (a1r – number of days elapsed until the female laid her first egg), early fecundity (F1-7 – number of eggs laid on the first week of life) and peak fecundity (F8-12 – number of eggs laid on the last five days of the assay). Around 12 mated pairs were assayed per population and mating type.

In the assay using groups a similar setup was applied, but this time with groups of 10 individuals per vial (5 females and 5 males, of the several mating types: s, m, ms and sm, grouped according with replicate number). In this assay, early fecundity was estimated as the number of eggs laid per vial divided by 5. To estimate peak fecundity we divided the number of eggs laid in each vial between days 8 and 12 by the number of females alive in that vial at the end of the assay, to reduce possible effects due to differential mortality. Sample size (in number of vials) was around 10 per population and mating type.

F_1 Hybrids

At generation 23 an assay was done involving a synchronous analysis of *D. madeirensis*, *D. subobscura* and their F1 hybrids (derived from the cross of same numbered *D. subobscura* and *D. madeirensis* replicate populations). This assay also allowed the comparison of 'F1 matings' with 'backcross matings'. F1 matings involved

F1 hybrid males and females derived from a given cross, while backcross matings involved mated pairs between F1 hybrids and parental species (with the same replicate number). Hybrids from both cross directions were assayed (msF1 hybrids with *D. madeirensis* as mother species and smF1 hybrids from the reciprocal cross). This assay involved the study of fecundity related traits like age of first reproduction, early and peak fecundity. Sample sizes were around 12 mated pairs for each population and mating type.

Statistical analysis

Behavior assays

For the proportion of mating pairs with courtship and courtships with mating bi-factorial ANOVAs with male and female as fixed factors with two categories (*D. madeirensis* and *D. subobscura*) were done on arcsine-transformed data (Sokal and Rolf 1995), estimated for each population and mating type. These analyses allowed testing for differences in male mating behavior and female receptivity for both species, as well as the effect of mating type on the expression of differences between species (tested by the interaction term). Several planned comparisons from one-way ANOVAS with type of mating as fixed factor, were carried out. In particular, the general difference between conspecific and heterospecific matings was tested. For all other planned comparisons, significance levels were adjusted for multiple testing with a sequential Bonferroni technique using available software (see Rice 1989).

For the temporal traits - courtship latency and duration, mating latency and duration - tri-factorial ANOVAs were performed, with female and male as fixed factors with two categories (*D. madeirensis* and *D. subobscura*) and replicate as random factor with three categories (1, 2 and 3), to test for the same effects as indicated above. The differences between species and both types of heterospecific matings were tested using planned comparisons from nested ANOVAs, replicate (1, 2, 3) nested in type of mating (s, m, ms and sm). In these analyses the term "replicate {type of mating}" was used as error, to properly estimate the significance level of the comparisons. Significance levels were adjusted with a sequential Bonferroni technique using available software (see Rice 1989), excluding the overall comparison between conspecific and heterospecific matings. All analysis were performed on transformed latencies and durations (ln x+1, Sokal and Rolf 1995).

Fecundity assays

Parental species

The arcsine transformed proportions of female mortality and of the number of egg-laying females in heterospecific and conspecific matings were compared using bi-factorial ANOVAs similar to the ones applied to courtship parameters from the behavioral assays. Planned comparisons from one-way ANOVAs with mating type as fixed factor were applied to compare both species and both types of heterospecific mating.

Tri-factorial ANOVAs with female and male as fixed factors with two categories each (*D. madeirensis* and *D. subobscura*) and replicate, a random factor with three categories (1, 2 and 3), were performed on all assayed traits (age of first reproduction, early fecundity and peak fecundity. This model allowed testing for differences in female performance between the two species, the effect of males from different species on female performance, and the effect of mating type on differences between species (tested by the interaction term).

The differences between species, both types of heterospecific matings as well as between conspecific and heterospecific matings were tested using planned comparisons from nested ANOVAs, as in the analysis of temporal traits in the behavior assays. In these analyses the term "replicate {type of mating}" was used as error, to properly estimate the significance level of the comparisons. Significance levels were adjusted with a sequential Bonferroni technique using available software (see Rice 1989), except for the comparison between conspecific and heterospecific matings.

F_1 Hybrids

The differences between parental species and hybrids were tested using planned comparisons from nested ANOVAs, with replicate as random factor (1, 2, 3) nested within mating type (m, s, F1sm and F1ms). A similar approach was used to test for differences in performance of hybrid females mated with hybrid males *versus* hybrid females mated with males from either parental species, this time with the ANOVA single factor having six categories (the two hybrid matings and the four possible backcross matings). Parental females mated with conspecific males (s, m) were also compared with parental females mated with hybrid males (s x F1ms, s x F1sm, m x F1ms, m xF1sm) using a similar approach. In all these analyses the term "replicate

{type of mating}" was used as error, to properly estimate the significance level of the comparisons.

Tri-factorial ANOVAs with female, fixed factor with two categories (msF1 and smF1), male, fixed factor with two categories (*D. subobscura* and *D. madeirensis*) and replicate, random factor with three categories (1, 2 and 3) were performed for all fecundity related traits. This analysis was performed to compare matings between female hybrids and males from both parental species using hybrids from both cross directions. A similar analysis was performed to compare F1 male hybrids from both cross directions mated with either parental species.

Results

Behavior

Conspecific crosses of *D. madeirensis* and *D. subobscura* presented a similar proportion of observed matings (m - 35 (44%), s – 32 (45%)). On the other hand, in both heterospecifc crosses fewer matings were observed, particularly in the heterospecific cross involving *D. madeirensis* females (ms – 4 (5%), sm - 19 (27%)).

The results of bi-factorial ANOVAs as well as of planned comparisons from one-way ANOVAs on proportion of pairs with courtship and proportion of courtships with mating are given on table I. Overall *D. madeirensis* females were less courted and mated less than *D. subobscura* ones. A significant male effect was also detected for both traits, the proportions of courting and mating *D. subobscura* males being lower. For the proportion of courtships with mating, the differences between the two species were higher in heterospecific than conspecific matings, as seen by the significant interaction term.

Drosophila madeirensis and *Drosophila subobscura* did not differ in either trait in conspecific matings. *D. madeirensis* males courted indiscriminately females from both species while *D. subobscura* males courted mainly conspecific females. Comparing females from both species gave similar indications. In general mating success was significantly higher when females from both species were mated with conspecific males. There was a significantly higher proportion of courtships with mating in conspecific pairs than in heterospecific ones (Table I).

Table I – Comparisons of *Drosophila madeirensis* and *D. subobscura* mated conspecifically and heterospecifically, for proportion of courtship and proportion of courtships with mating.

Effect	**courtship**	**mating/courtship**
female	8.090*	6.441*
male	5.489*	7.611*
female*male	3.708 n.s.	62.924***
Contrasts		
consp. *vs*. heterosp	3.708 n.s.	62.924***
m *vs.* s	0.126 n.s.	0.024 n.s.
ms *vs.* sm	13.452**	14.028*
m *vs.* ms	9.110*	57.151***
m *vs.* sm	0.422 n.s.	14.550*
s *vs.* ms	11.376**	54.815***
s *vs.* sm	0.087 n.s.	13.384*

F- values of bi-factorial ANOVAs with male and female, both fixed factors with two categories (*D. madeirensis* and *D. subobscura*) on the arcsine transformed proportions of mating pairs with courtship and courtships with mating. The *F*- values of planned comparisons from one-way ANOVAs with mating type as fixed factor with four categories (both conspecific matings (m – *D. madeirensis*, s – *D. subobscura*) and both heterospecific matings (ms - ♀♀ *D. madeirensis* x ♂♂ *D. subobscura*, ♀♀ *D. subobscura* x ♂♂ *D. madeirensis*) are also given. n.s. – non significant, * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$.

The analysis of the effect of mating types on temporal traits must be seen with caution given the very small sample sizes, particularly for the cross *Drosophila madeirensis* females x *Drosophila subobscura* males (see above). When comparing mating types for temporal traits no differences were found between species (male and female effects from the ANOVA analyses gave no significance) and the interaction term was only significant for courtship latency. This was due to the fact that *D. subobscura* males presented the bigger courtship latency in the interspecific cross, and the smaller in conspecific matings (Table 2, Figure 1).

Except for mating duration, planned comparisons gave no indication of significant differences for temporal traits, between conspecific and heterospecific matings in general, as well as for each species (Table II). Males from both species took a similar amount of time to recognize conspecific and heterospecific females as

potential mating partners. Also, the time spent courting and until mating occurred was similar for conspecific and heterospecific matings. Mating duration, on the other hand, was shorter in both heterospecific matings than within either species (Table II, Figure 1).

Table II – Comparisons of *Drosophila madeirensis* and *D. subobscura* mated conspecifically and heterospecifically for temporal behavioral traits.

Effect	**Courtship latency**	**Courtship duration**	**Mating latency**	**Mating duration**
female	0.549 n.s.	0.968 n.s.	1.174 n.s.	2.144 n.s.
male	2.122 n.s.	1.962 n.s.	0.112 n.s.	0.019 n.s.
female*male	57.429*	0.066 n.s.	5.555 n.s.	15.143 m.s.
Contrasts				
consp. *vs* heterosp.	1.509 n.s.	0.083 n.s.	2.284 n.s.	36.469**
m *vs.* s	0.032 n.s.	0.003 n.s.	1.120 n.s.	1.687 n.s.
ms *vs.* sm	0.841 n.s.	1.941 n.s.	0.806 n.s.	0.353 n.s.
m *vs.* ms	1.423 n.s.	0.722 n.s.	1.226 n.s.	12.206**
m *vs.* sm	0.103 n.s.	0.774 n.s.	2.478 n.s.	37.111**
s *vs.* ms	1.829 n.s.	0.790 n.s.	1.238 n.s.	16.474**
s *vs.* sm	0.253 n.s.	0.900 n.s.	0.078 n.s.	26.228**

F- values of tri-factorial ANOVAs with female, male (both fixed factors) and replicate (random factor) for each temporal trait and for contrasts comparing the several types of conspecific and heterospecific matings from nested ANOVAs (replicate {couple}) m- conspecific matings of *D. madeirensis*, s - conspecific matings of *D. subobscura*, ms - ♀♀ *D. madeirensis* x ♂♂ *D. subobscura*, sm - ♀♀ *D. subobscura* x ♂♂ *D. madeirensis*. n.s. – non significant, m.s. 0.05< p < 0.06, * p < 0.05, ** p < 0.01, *** p < 0.001.

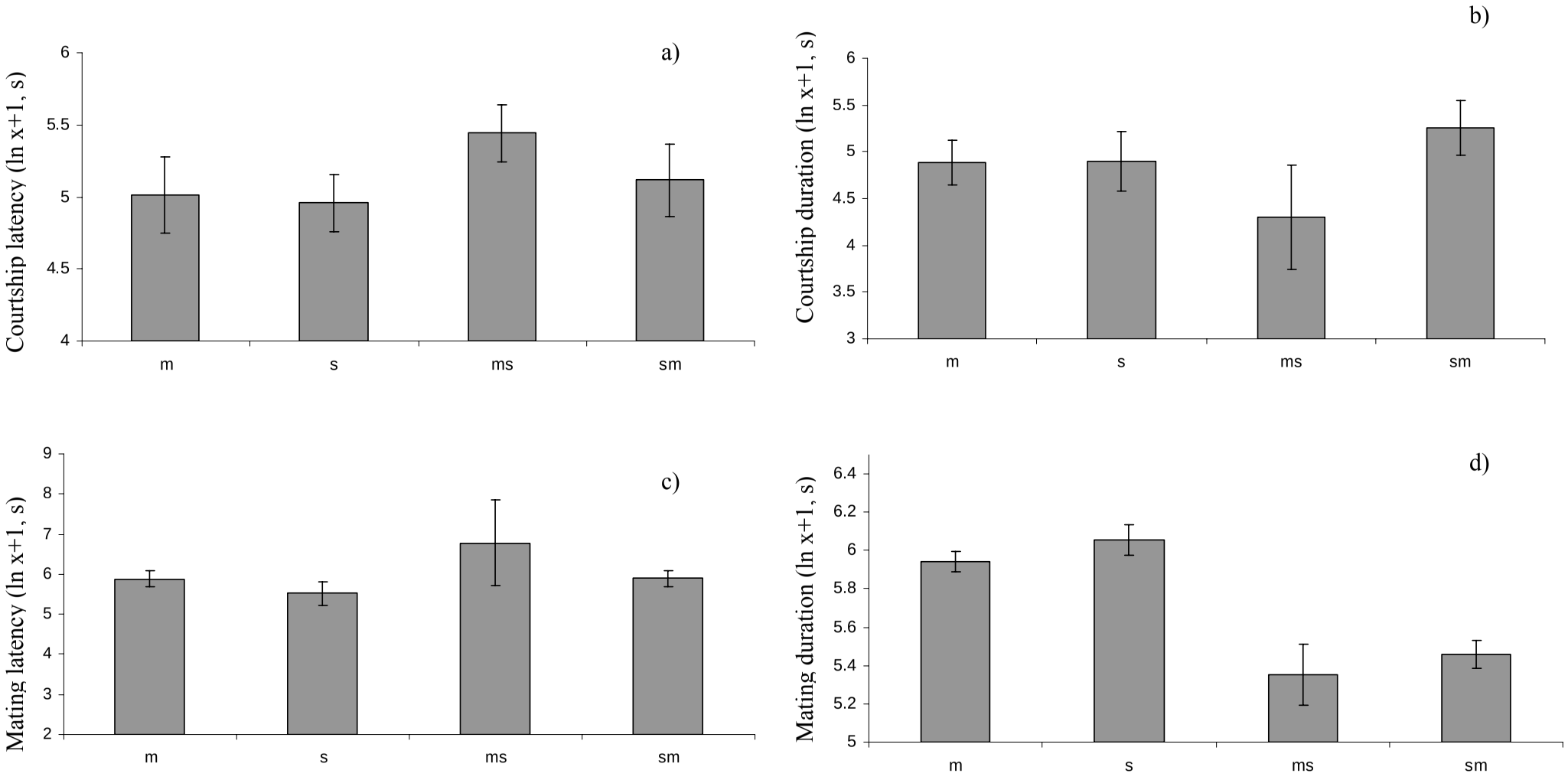

Figure 1 – Average values (ln x+1, s) for behavioral traits for conspecific and heterospecific matings (m - *D. madeirensis* and s - *D. subobscura*).

Fecundity

Assay with pairs

The results from bi-factorial ANOVAs indicate that *Drosophila madeirensis* and *Drosophila subobscura* did not differ in female mortality during the assay, either in conspecific or heterospecific matings, as none of the factors (male, female) or the interaction term was significant (data not shown).

A high number of females mated conspecifically laid eggs in both species (m – 33 (92%), s- 30 (85%)), while females mated with heterospecific males showed a poor performance, particularly *D. madeirensis* females (ms – 8 (28%), sm – 22 (61%)). Comparing the number of females which laid eggs gave indication of significant male effects with an average better performance of *D. madeirensis* males (F = 5.81, $p < 0.05$). The interaction term was also significant indicating that the two species differed in their relative performance when mated with conspecifics *versus* heterospecifics (F = 15.020, $p < 0.01$), *D. madeirensis* females being more affected by mating with heterospecific males.

Planned comparisons indicate that the two species in conspecific matings did not differ in number of egg-laying females (data not shown), and that *D. madeirensis* females mated with *D. subobscura* males laid fewer eggs (F = 6.164, $p < 0.05$) than the reciprocal cross.

Tri-factorial ANOVAs indicate that age of first reproduction was the only trait presenting differences in this assay. Females' performance for this trait was affected by the males with whom they mated, *D. madeirensis* females mated with *D. subobscura* males starting to lay eggs later than any other mating type (Table III, Figure 2).

The results from planned comparisons show that, after sequential Bonferroni correction, the two species presented similar values for all analyzed traits in this assay (Table III). On the other hand, the two reciprocal heterospecific matings differed significantly in most traits, the cross direction involving *D. madeirensis* females presenting a lower performance in all fecundity related traits (Figure 2, Table III). Conspecifically and heterospecifically mated females differed significantly for age of first reproduction and peak fecundity, conspecifically mated females showing, in general, a better performance. For peak fecundity the difference was mainly due to the interspecific cross involving *D. madeirensis* females, which laid fewer eggs (Figure 2, Table III).

Table III - Comparisons of *Drosophila madeirensis* and *D. subobscura* mated conspecifically and heterospecifically for fecundity traits, in the assays involving pairs and groups of individuals.

Pair assay

Effect	**Age of first reproduction**	**Early fecundity**	**Peak fecundity**
female	2.59 n.s.	10.91 n.s.	13.31 n.s.
male	33.90*	2.08 n.s.	0.84 n.s.
female*male	31.36*	8.61 n.s.	8.41 n.s.
Contrasts			
consp. *vs.* heterosp.	27.12**	5.86 n.s.	14.31*
m vs s	0.11 n.s.	6.51 n.s.	2.09 n.s.
ms vs sm	8.29*	3.07 n.s.	6.35*
m vs ms	27.48**	1.86 n.s.	10.43*
m vs sm	5.99*	0.19 n.s.	0.48 n.s.
s vs ms	23.55**	14.25*	21.12**
s vs sm	4.32 n.s.	4.25 n.s.	4.45 n.s.

Group assay

Effect	**Age of first reproduction**	**Early fecundity**	**Peak fecundity**
female	20.87*	83.02*	56.96*
male	0.58 n.s.	12.34 n.s.	3.64 n.s.
female*male	45.88*	473.64**	1261.72***
contrasts			
consp. *vs.* heterosp.	9.93*	81.32***	43.50***
m vs s	17.79*	140.33***	40.11***
ms vs sm	27.51**	29.74**	48.42***
m vs ms	7.50*	10.27*	24.84**
m vs sm	6.50*	5.24 n.s.	4.04 n.s.
s vs ms	492.16***	219.43***	124.57***
s vs sm	2.95 n.s	91.72***	18.84**

F-values and the corresponding significance levels from each tri-factorial ANOVA. The results of planned comparisons from nested ANOVAs and their significance levels comparing species and types of heterospecific matings, are also given. See Table II for more details. n.s. – non significant, m.s. - $0.05 < p < 0.07$ * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

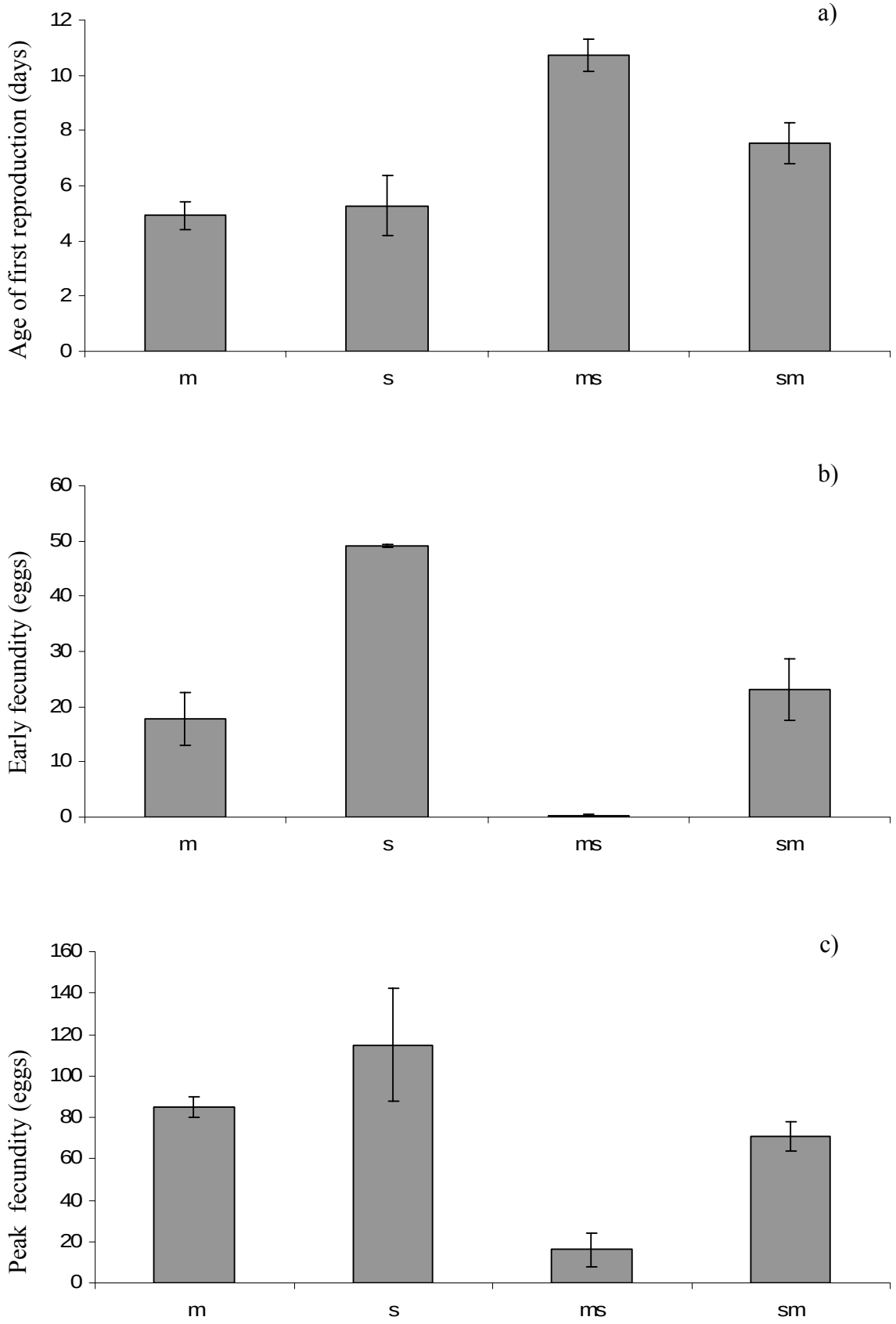

Figure 2 – Average values of fecundity related traits for conspecific and heterospecific matings (m - *D. madeirensis* and s - *D. subobscura*) at generation 7.

Assay with groups

Of the 144 vials analyzed in this assay, 4% did not yield any eggs during the entire assay, all from matings between *D. madeirensis* females and *D. subobscura* males.

Females from the two species differed significantly for all assayed traits, with the same tendency as the one found in the assay with pairs: *D. subobscura* females started to lay eggs earlier and were more fecund (Figure 3, Table III). Differences between females also changed significantly depending on the male with which they mated, with the best performance being the conspecific *D. subobscura* matings and the lowest the heterospecific mating involving *D. madeirensis* females. Considering planned comparisons, conspecific crosses performed better, as in the assays with pairs. Female *D. madeirensis* mated with male *D. subobscura* started to lay eggs significantly later and had lower fecundity than the opposite cross direction. In general, *D. subobscura* females mated with conspecific males had the best performance of all crosses. The interspecific cross involving *D. subobscura* females had a similar performance in fecundity as the conspecific cross of *D. madeirensis*.

Hybrids vs. parental species

At generation 23 the F1 hybrids from both cross directions were included in the assay of fecundity related traits. The results from planned comparisons indicate that in this generation, *D. madeirensis* and *D. subobscura* differ in all traits except age of first reproduction, with *D. subobscura* being more fecund. The two reciprocal hybrids presented similar values for all analyzed traits. When compared with parental species hybrids were less fecund. This difference was mainly due to a lower performance relative to *D. subobscura* (Table IV).

Hybrid females mated with hybrid males ('F1 matings') started to lay eggs significantly later and were less fecund than female hybrids mated with parental males ('backcross matings'). Parental females mated with conspecific males started to lay eggs earlier and differed significantly in fecundity from parental females mated with hybrid males (Table IV). This was due to the fact that *D. subobscura* females mated conspecifically laid more eggs than any other type of mating (Figure 3).

Tri-factorial ANOVAs were performed comparing both types of hybrid females mated with each parental species. The results indicate that female hybrids from both cross directions present similar performances when mated with either parental male for

all analyzed traits (data not shown). Age of first reproduction was the only trait where a significant male effect was detected ($F = 77.94$, $p < 0.01$), mainly due to the fact that F_1sm hybrid females had a lower age of first reproduction when mated with *D. madeirensis* males.

When comparing the performance of parental females mated with hybrid males, using a similar analysis, significant differences between females from the two species were found in both fecundity periods (F1-7 – $F = 134.96$, $p < 0.01$; F8-12 – $F = 191.53$, $p < 0.01$) but not in age of first reproduction. *D. madeirensis* females in general had a poorer performance when mated with either type of hybrid male.

Table IV – ANOVA results comparing conspecific matings for both species and both types of F_1 matings.

	Age of first reproduction	**early fecundity**	**peak fecundity**
m *vs* s	2.439 n.s.	87.224***	82.671***
F_1ms *vs* F_1sm	0.216 n.s.	0.009 n.s.	3.176 n.s.
P *vs* F_1	31.485 n.s.	45.064***	10.021*
F_1 *vs.* F_1P	7.970*	25.528***	54.068 n.s.
P *vs.* PF_1	68.624***	54.068***	34.334***

F- values and significance levels of planned comparisons from nested ANOVAs, with replicate populations nested in mating type, comparing hybrids and parental species. The comparisons between hybrid females mated with hybrid males (F_1) and mated with parental males (F_1P) as well as parental females mated conspecifically (P) and with hybrid males (PF_1) are also given. F_1*ms* – F_1 hybrid having *D. madeirensis* as the mother species, F_1*sm* – F_1 hybrid from the reciprocal cross, F_1 – F_1 hybrids, P – parental species. Significance levels are also given. n.s. – non significant, * $p < 0.05$, **$p < 0.01$, *** $p < 0.001$.

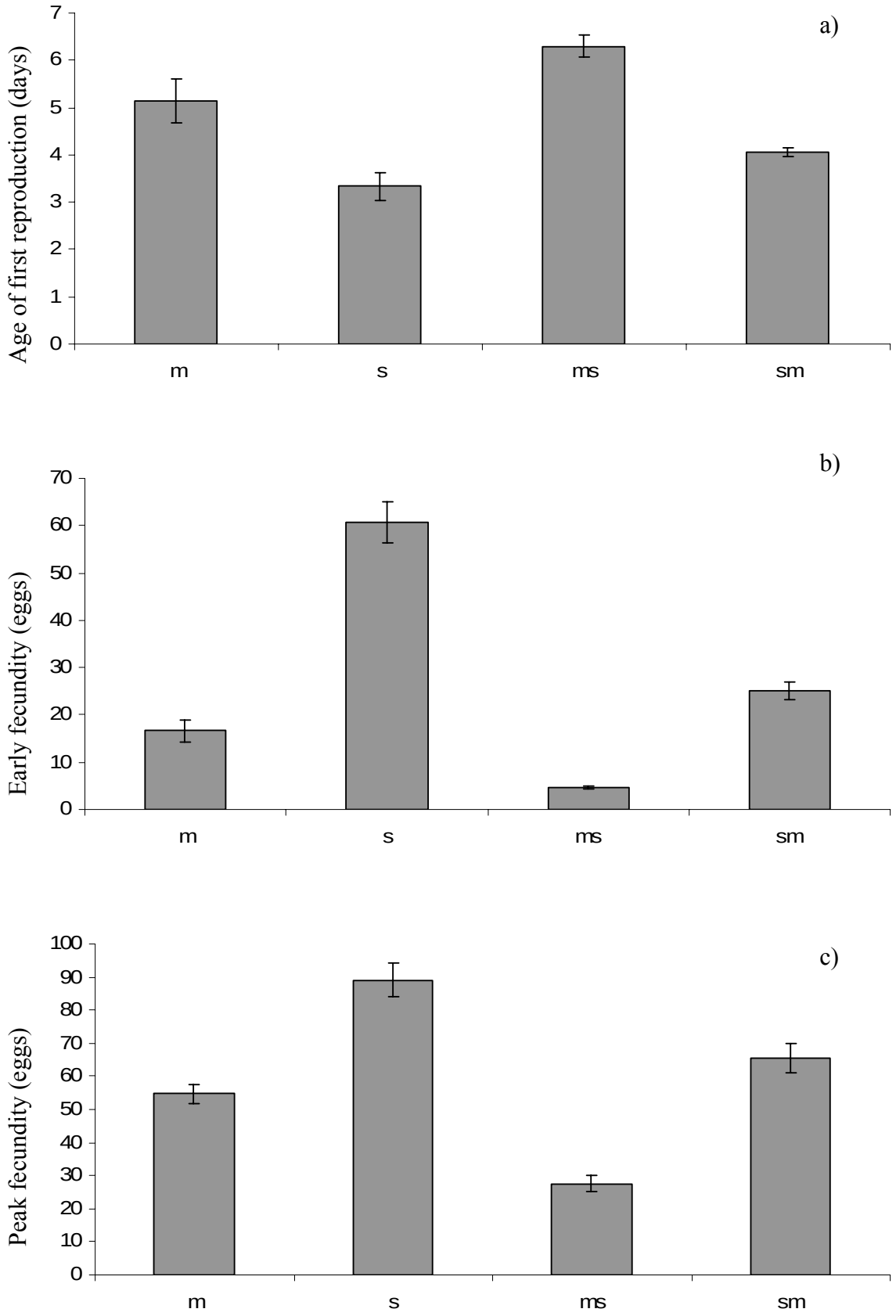

Figure 3 - Average values of fecundity related traits for groups of conspecific and heterospecific matings (m - *D. madeirensis* and s - *D. subobscura*) at generation 11.

Discussion

Assortative mating between D. madeirensis and D. subobscura

Drosophila madeirensis and *Drosophila subobscura* present assortative mating, conspecific matings being more likely than heterospecific ones, particularly considering the crosses involving *Drosophila madeirensis* females. In fact, there were so few cases of this cross with courtship (27%) and courtship with mating (5%) that this limited our statistical power to detect differences in mating behavior involving the more detailed, temporal analysis. We will thus center our discussion in the results involving proportions of pairs with courtship and courtships with mating.

One might think that the time allowed for mating to occur (45 minutes) was not sufficient for some *D. subobscura* males to recognize *D. madeirensis* females as suitable mating partners. Nevertheless, our fecundity assay using pairs (see below) also reveals that a high proportion of females in this type of cross (72%) did not lay any eggs throughout the first 12 days of potential contact, indicating that even long periods did not lead to effective mating in this cross direction.

Heterospecific matings between these species show asymmetry, with the direction involving *D. madeirensis* males being more probable than the reciprocal one. Asymmetry in heterospecific matings has been linked with evolutionary history but this association has been controversial (Kaneshiro 1976; 1980; Watanabe and Kawanishi 1979; Markow 1981; Fraser and Boake 1997; Ödeen and Florin 2002). Kaneshiro's (1976; 1980) hypothesis states that derived males are not accepted by ancestral females. Watanabe and Kawanishi (1979) claim the opposite, namely that derived females discriminate against ancestral males.

The asymmetry observed between *D. madeirensis* and *D. subobscura* seems to indicate that females from the derived species (*D. madeirensis*) "discriminate" against ancestral males, as shown by the significantly smaller proportion of courtships that resulted in mating in that cross direction. This is in accordance with Watanabe and Kawanishi's hypothesis of reproductive isolation and contradicts the one proposed by Kaneshiro. However, it is possible that the different size between the two species (with *D. madeirensis* bigger than *D. subobscura*, see Rego et al. 2006) may have contributed to the general better performance of interspecific matings involving *D. madeirensis* males. Several studies indicate that bigger males have better mating success in

Drosophila, at least in intraspecific crosses (see Monclús and Prevosti 1971 in *D. subobscura*; see Partridge et al. 1987; Santos et al. 1988; James and Jaenike 1992 for other species).

It has been suggested that measures of reproductive isolation and assortative mating can be influenced by differences between species in mating propensity (e.g. Markow 1981). However, we did not find differences in mating propensity between *D. madeirensis* and *D. subobscura*, in conspecific matings, as shown by similar proportions of courtship, courtship with mating, and temporal traits.

The mating asymmetry between the two interspecific cross directions could either reflect that *D. madeirensis* females are more discriminating, or that *D. subobscura* males are less attracted by heterospecific females. Courtship latency is considered as an indicator of how long the male takes to identify a female as a potentially receptive mate (Noor 1996); while courtship duration is probably an indicator of female receptivity. Our data indicate that the two interspecific crosses did not differ for any of these traits. However, as we have already pointed out, our analysis of temporal traits excluded couples where courtship was not observed, the majority of which (53%) were between *D. madeirensis* females and *D. subobscura* males. In this respect the data on proportion of pairs with courtship and of courtships with mating are more robust, and suggest that both males and females may have contributed to the differences between the two cross directions, corresponding to a smaller interest of *D. subobscura* males and reduced receptivity of *D. madeirensis* females, in interspecific matings. *D. madeirensis* males, on the other hand, court similarly females from both species.

Evidence of males courting indiscriminately conspecific and heterospecific females has also been found in both species of the species pair *D. pseudoobscura* - *D. persimilis* (Noor 1996). These species also belong to the *obscura* group but they diverged more recently (see Ramos-Onsins et al. 1998), which seems in accordance with the absence of discrimination, since mating discrimination increases with divergence time (Coyne and Orr 1989). It has been suggested that no-choice tests, like the ones used in our assays, may be insufficient to determine whether males are really indiscriminate (Gupta and Sundaran 1994; Wu et al. 1995). Nevertheless, choice and no-choice tests using *D. pseudoobscura* and *D. persimilis* gave similar results (Noor 1996).

Does mating with heterospecifics have reproductive costs?

A basic expectation of evolutionary theory is that, because in general females invest more in offspring, producing few larger gametes, they have to choose their mating partner to guarantee that their investment has a successful outcome (Andersson 1994; Shuster and Wade 2003).

Mating with heterospecifics can have significant reproductive costs namely on fecundity (e.g. Wade et al. 1994; Sota and Kubota 1998; Shapiro 2000; Price et al. 2001). Our fecundity data are in accordance with this expectation. Females mated conspecifically had a better performance in fecundity related traits than females mated with males from the other species. The cost of mating on fecundity is particularly evident in *D. subobscura* females: the difference between species in conspecific matings (with *D. subobscura* having a better performance; see also Rego et al. 2007a) disappears when both mate with *D. madeirensis* males.

In general, the assay using groups revealed more significant differences between species, either in conspecific matings or in differences with heterospecific matings. This is due, at least in part, to a higher statistical power in that assay derived from the higher number of females assayed.

Lower fecundity in heterospecific matings has also been found in other species: mites (Collins and Margolies 1991), crickets (Tanaka 1991), flour beetles (Wade et al. 1994), carabids (Sota and Kubota 1998), katydids (Shapiro 2000), *Drosophila* (Price et al. 2001).

One cause for lower fecundity in heterospecific matings may be lower fertilization. For instance, mating duration could be insufficient for sperm transfer to be completed and reduced oviposition in heterospecific matings could reflect reduced fertilization (e.g. Price et al. 2001). However, this is not plausibly the case here. Though mating duration was significantly lower for heterospecific matings in either direction than for conspecific ones, it was still probably sufficient for sperm transfer to be successful. In several species from the *obscura* group, sperm transfer starts within 1.5 minutes after the beginning of copulation (Snook 1998). The heterospecific matings we observed lasted on average 3.51 ± 0.03min for matings involving *D. madeirensis* females and 5.10 ± 0.25min for the reciprocal cross. Thus it is reasonable to assume that both heterospecific crosses had a sufficient duration to allow sperm transfer.

Another possible explanation for reduced fecundity in heterospecifc matings could be related with the fact that in some cases the female can discard the heterospecific sperm (e.g. Price et al. 2001). Finally, it is also possible that in heterospecific matings the foreign seminal proteins are unable to stimulate oviposition, contrary to the stimulation that may occur in conspecific matings (e.g. Herndon and Wolfner 1995 in *Drosophila melanogaster).*

Hybrids and implications for speciation

The reproductive isolation between *D. madeirensis* and *D. subobscura* is incomplete. Interspecific crosses are possible and under laboratorial conditions it is possible to obtain viable and fertile female and male hybrids from both cross directions, especially if the mother species is *D. madeirensis* (Rego et al. 2007b). This contrasts with behavior observations, which indicate that the reciprocal cross is more probable. These contrasting results suggest that different mechanisms may have evolved to prevent the detrimental effects of hybridization in the two cross directions between these species. In one cross direction the reproductive barrier seems to be pre-zygotic and mainly behavioral (*D. madeirensis* females x *D. subobscura* males), maintaining some genetic compatibility, and allowing the production of hybrids when behavioral barriers are broken. The hybrid progeny resulting from this cross direction presents an even sex-ratio (Khadem & Krimbas 1993; Rego et al. 2006; 2007b). On the other hand, in the reciprocal cross apparently the barrier is mainly post-zygotic, since *D .madeirensis* males readily courted and mated with *D. subobscura* females. This interspecific cross is easily observed under laboratorial conditions and curiously yields male-biased progeny (Khadem and Krimbas 1991; Papaceit et al. 1991; Rego et al. 2006; 2007b). This contradicts Haldane's rule, which states that it is the heterogamic sex (in this case the males) which is expected to suffer the most detrimental effects from hybridization. In fact, though Haldane's rule is well supported by empirical evidence, the genetic mechanisms responsible for its occurrence are not completely understood (reviewed in Markow et al. 2007). Interestingly, we have had other unexpected results in the context of Haldane's rule, with female hybrids from the cross *D. madeirensis* females and *D. subobscura* males presenting higher developmental asymmetry than the parental species while hybrid males do not (Rego et al. 2006).

Our findings question how reproductively isolated *Drosophila subobscura* and *Drosophila madeirensis* are. As we saw in this and previous studies, crosses between the two species entail reproductive costs that are asymmetric in the two reciprocal cross directions. Other data indicate that F_1 hybrids between *D. subobscura* and *D. madeirensis* are viable and partially fertile (Rego et al. 2007b) and that F2 hybrids are fertile (C. Rego, unpublished results). As the F_1 hybrids present lower fitness relative to pure species in terms of fecundity, backcrosses would be a possible way by which natural hybridization between these species could influence their future evolution. This is particularly true in the case of the interspecific cross involving *D. subobscura* females, as this cross yields mostly males. Nevertheless, we have to be cautious in extrapolating results from laboratorial trials to what really can happen in nature (e.g. Harshman and Hoffmann 2000; Llopart et al. 2005). Being able to obtain hybrids in the laboratory does not mean that hybridization occurs in nature, and even when natural hybrids are found they do not necessarily conform to the expectations from laboratorial trials. Recent data on a hybrid zone involving *D. yakuba* and *D. santomea* reinforce these limitations. This hybrid zone revealed an unexpected finding: the hybrids observed in nature were not from the cross direction expected from laboratorial trials but from the reciprocal one (Llopart et al. 2005).

The possible occurrence of natural hybrids between *D. madeirensis* and *D. subobscura* has been reported (Khadem et al. 2001). So it would be very interesting to investigate in more detail the occurrence of natural hybrids and/or backcrosses to ascertain their possible importance in the future evolution of *D. madeirensis* and *D. subobscura*. Specifically, it would be important in the future to study how frequently hybrids between the two species occur in nature, in particular taking into account that *Drosophila madeirensis* is an endemic species of Madeira, associated with the Laurisilva. This type of habitat is currently being threatened by human activities. Habitat reduction and fragmentation is one possible way in which hybridization could be facilitated (e.g. Rhymer and Simberloff 1996; Allendorf et al. 2001), by promoting encounters between the two species, by reducing the number of conspecifics available for reproduction, and even by creating intermediate habitats (Laurisilva fragment edges) where hybrids could have some reproductive success. Thus hybridisation could accelerate the extinction of yet another unique species.

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Chapter 4.

Symmetry breaking in *Drosophila* hybrids is not due to developmental noise

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SYMMETRY BREAKING IN INTERSPECIFIC *DROSOPHILA* HYBRIDS IS NOT DUE TO DEVELOPMENTAL NOISE

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Abstract.—Hybrids from crosses of different species have been reported to display decreased developmental stability when compared to their pure species, which is conventionally attributed to a breakdown of coadapted gene complexes. *Drosophila subobscura* and its close relative *D. madeirensis* were hybridized in the laboratory to test the hypothesis that genuine fluctuating asymmetry, measured as the within-individual variance between right and left wings that results from random perturbations in development, would significantly increase after interspecific hybridization. When sires of *D. subobscura* were mated to heterospecific females following a hybrid half-sib breeding design, F_1 hybrid females showed a large bilateral asymmetry with a substantial proportion of individuals having an asymmetric index larger than 5% of total wing size. Such an anomaly, however, cannot be plainly explained by an increase of developmental instability in hybrids but is the result of some aberrant developmental processes. Our findings suggest that interspecific hybrids are as able as their parents to buffer developmental noise, notwithstanding the fact that their proper bilateral development can be harshly compromised. Together with the low correspondence between the covariation structures of the interindividual genetic components and the within-individual ones from a Procrustes analysis, our data also suggest that the underlying processes that control (genetic) canalization and developmental stability do not share a common mechanism. We argue that the conventional account of decreased developmental stability in interspecific hybrids needs to be reappraised.

Key words.—Canalization, developmental stability, *Drosophila madeirensis*, *Drosophila subobscura*, fluctuating asymmetry, geometric morphometrics, interspecific hybrids, quantitative genetics.

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Hybrids between divergent lineages quite often have reduced viability and/or fertility as a result of unfavorable interactions between the genomes of the parental types. By far the best evidence on the role of gene interactions on those traits comes from *Drosophila*, particularly from the *D. melanogaster* complex hybrids (for a review, see Coyne and Orr 2004). In contrast to the rich literature focused on localizing the alleles associated with failed gene interactions, very little is known about the developmental basis of hybrid problems, including the incidence of morphological abnormalities in some interspecific crosses. The finest example comes again from *Drosophila*, where *D. melanogaster–D. simulans* hybrid lethality probably involves a cell-cycle defect that prevents the formation of imaginal discs (Orr et al. 1997): those larval structures in holometabolous insects hosting undifferentiated cells that will differentiate during the pupal phase into adult structures.

To understand the negative effects of hybridization on development a potentially rewarding line of research comes from recent progress in canalization, developmental stability, and functional genomics. Canalization describes the ability of the genome to repress phenotypic variation as a result of genetic (genetic canalization) or environmental (environmental canalization) disturbances (Waddington 1957; Gibson and Wagner 2000; Debat and David 2001). Developmental stability is the ability of organisms to buffer against the random noise that arises spontaneously as a consequence of stochastic variation in the cellular processes that are involved in the development of morphological structures (Klingenberg 2004). Albeit still a controversial issue, both canalization and developmental stability are subcategories of buffering mechanisms that can be partially overlapping (e.g., Dworkin 2005a,b; Santos et al. 2005; Willmore et al. 2005). Also contentious is the negative effect of hybridization on developmental stability, which to a certain extent may be due to difficulties associated with obtaining an unbiased estimate of underlying developmental noise (Markow 1995; Alibert and Auffray 2003; Pélabon et al. 2004).

The most commonly used estimate of developmental stability in bilaterally symmetrical organisms is fluctuating asymmetry (FA), the intraindividual variation between right and left sides, which is generally assumed to be a proxy for the developmental noise of the organism. If the only real cause of asymmetry is variation due to stochasticity in development, then FA can indeed be taken as an estimate of developmental stability. Another source of asymmetry is directional asymmetry (DA), which is fairly common and occurs when there is a consistent difference between left and right body sides. Because this source of asymmetry is not a measurement of developmental stability (Klingenberg 2003), confounding estimates involving FA and DA should be avoided (Palmer 1994; Palmer and Strobeck 2003). However, even ideal FA (i.e., a normal distribution of right-left scores whose mean is zero; but for a cautionary note on the overstressed importance of normal distributions in FA studies see Klingenberg 2003, pp. 22–23) is not warranty for the absence of genetic variation in DA as there can be genetic variation for DA in traits that exhibit nonsignificant DA (Leamy et al.

1997). This may, in turn, have an effect on FA (Santos 2002). In addition, while the molecular mechanisms underlying asymmetry of internal organs are being deciphered (see Palmer 2004; Raya and Izpisúa Belmonte 2004; Levin 2005), it has been a tenet in FA research that external right-left symmetry is the default state and needs no further explanation. Astonishingly enough, this default idea has been recently rebuffed by evidence suggesting that symmetry-generating mechanisms do seem to be necessary for a proper development (see Hornstein and Tabin 2005). Interspecific hybridization does seem to enhance asymmetry (Alibert and Auffray 2003, table 8.3), which suggests that epistatic effects can be important in controlling FA levels (Leamy and Klingenberg 2005). Nevertheless, it is by no means clear whether this could be due to an increase of developmental instability in hybrids or to a disruption of symmetry-generating mechanisms when different coadapted genomes are combined.

From a more mechanistic point of view, a key question is the molecular meaning of randomness in development. Left and right body sides share the same genome (barring unusual somatic mutation or somatic recombination) and in most organisms very nearly the same environment, but the inherently stochastic nature of gene expression—transcription in eukaryotic cells has been described as quantal, with pulses of mRNA produced in a probabilistic manner—can easily translate into phenotypic variation (see Blake et al. 2003; Nijhout 2004; Kærn et al. 2005) and significantly contribute to FA. The level of gene expression noise in eukaryotic cells is strongly influenced by transcription (e.g., Becskei et al. 2005), which in connection with interspecific hybridization and current developments in functional genomics could be used to elucidate important properties of development stability. For instance, recent analyses of gene expression patterns in *Drosophila* hybrids versus pure species have underscored hybrid disruptions and quantitative misexpression of genes associated with those hybrid dysfunctions (e.g., Michalak and Noor 2003; Ranz et al. 2004; Noor 2005). It would be interesting to relate this kind of finding with levels of developmental instability because misexpression of genes in hybrids could lead to increased FA.

Here we use two closely related *Drosophila* species to test the hypothesis that hybridization enhances (genuine) developmental instability. *Drosophila madeirensis* is endemic to Madeira Island, where it coexists with its close relative and widespread species *D. subobscura*. The two species are morphologically rather similar (Monclús 1984) and their reproductive isolation is not complete, as viable and fertile hybrids (only females; but see below) are obtained in some interspecific crosses (Khadem and Krimbas 1991, 1993; Papaceit et al. 1991). According to nucleotide divergence at the *rp49* gene region these species diverged about 0.6–1 million years ago (Ramos-Onsins et al. 1998), but the ancestors of *D. madeirensis* and extant *D. subobscura* populations in Madeira are probably the result of independent colonization events from the continent (Khadem et al. 1998). When *D. madeirensis* females are crossed to *D. subobscura* males, the interspecific hybrids show the following three traits: (1) a relatively large number of progeny can be produced and the sex ratio of hybrid families does not greatly deviate from 1:1; (2) both female and male hybrids display some anomalous phenotypic traits, such as deformed head shape and abnormalities of abdominal tergites; and (3) hybrid males also express the extra sex comb (ESC) phenotype with incomplete penetrance (Papaceit et al. 1991; Khadem and Krimbas 1993). In the reciprocal cross (*D. subobscura* females × *D. madeirensis* males), the sex ratio of hybrid families is greatly male biased (varying from 30:1 to 1.2:1) with fertile females and phenotypically normal but otherwise sterile males (Khadem and Krimbas 1991, 1993, 1997; Papaceit et al. 1991). These findings do not squarely conform to Haldane's (1922) rule, which states that, in interspecific hybridizations, the F_1 hybrids of the heterogametic sex (the males in this case) are more adversely affected than hybrids of the homogametic sex. The *D. subobscura–D. madeirensis* asymmetry for hybrid inviability provides clear evidence for the role of maternally expressed genes (Turelli and Orr 2000).

Because of the aforementioned incidence of hybrid morphological abnormalities in *D. madeirensis–D. subobscura* crosses (throughout this paper the maternal species is always indicated first), this system seems quite appropriate to also investigate the extent of congruence between canalization (which can be appraised by estimating interindividual variance) and FA when coadapted genomes are disrupted. By applying the methods of geometric morphometrics (Bookstein 1991, 1996; Dryden and Mardia 1998; Adams et al. 2004; Zelditch et al. 2004) to the wing vein network, we report here that F_1 female hybrids have increased levels of wing size bilateral asymmetry when compared to their parental species. However, after partitioning out the genetic component of right-left variation from a *D. madeirensis–D. subobscura* hybrid half-sib breeding design (i.e., from a decomposition of the individual × side interaction effect in the conventional mixed model for the study of right-left asymmetries into causal components attributable to DA), the levels of environmental intraindividual variation in those hybrids were found to be analogous to those for pure species FAs. The higher asymmetry was basically due to a substantial increase in among-sire variance for DA. In addition, a low congruence between the covariation structures of the interindividual genetic components and the intraindividual ones from a Procrustes analysis was found. Regardless of showing important morphological abnormalities, the results challenge the conventional prospect that interspecific hybrids are expected to show higher levels of developmental instability.

Materials and Methods

Fly Stocks and Handling

The *D. madeirensis* and *D. subobscura* base stocks were established in April 2001 from a sample of wild flies collected in a patch of Laurissilva forest near Ribeiro Frio (Madeira Island; 32°43′N, 16°52′W). The populations have been maintained on a discrete generation (of 30 days each), controlled larval and adult crowding regime at 18°C (12:12 light:dark cycle; for details see Matos et al. 2000, 2002). Population sizes were kept around 1000 individuals, never dropping below 400. At the time of this assay the flies had been maintained in the laboratory for more than 35 generations.

We note here that, contrary to what had been previously reported, our populations do produce fertile F_1 hybrid males

in both directions (i.e., *madeirensis* × *subobscura* and *subobscura* × *madeirensis*), and F_2 hybrids from both crosses can be obtained. In addition, the abnormal head trait is almost absent, and the males tend only to express the ESC phenotype (C. Rego, unpubl. data).

Hybrids from ♀♀ *D. madeirensis* × ♂ *D. subobscura* crosses (hereafter referred to as *DmDs*) were obtained following a paternal half-sib mating design. Thus, 82 one-week-old *D. subobscura* males were individually crossed in vials to eight *D. madeirensis* virgin females of the same age. After one week the vials were visually inspected for the presence of eggs, and all females from those vials containing eggs were individually placed in fresh vials for further egg laying. Offspring hybrids were then obtained from each inseminated female after transferring a sufficient number of eggs for larval development to new vials with abundant food. Only 66 (80%) of the initial 82 sire families produced offspring, and the number of females with progeny varied between one and seven per male (average ± SE: 4.32 ± 1.12). Twelve crosses were further discarded from the analyses because just a single dam produced offspring; the remaining 54 families rendered a total of 1028 females and 888 males. The number of offspring per dam ranged from one to 44 (10.51 ± 6.18) with an average sex ratio of 0.44 ± 0.19. A close inspection of the data suggests that there is no apparent relationship between an even sex ratio and a higher number of progeny.

Hybrids from the reciprocal cross (i.e., *DsDm*) were also analyzed, but due to the low productivity of these crosses it was unfeasible to perform a hybrid half-sib breeding design and, therefore, F_1 hybrids were obtained from mass crosses. A total of 250 *D. subobscura* virgin females were crossed to the same number of *D. madeirensis* virgin males, and the flies were maintained in the same conditions as the parental populations. Only the male progeny was analyzed because of the extremely biased sex ratio observed.

Both the pure species and the interspecific hybrids from the half-sib breeding design and the mass crosses were fixed in a 3:1 mixture of alcohol and glycerol at 4°C before wing measurements. All fly handling was done at room temperature (22–24°C), using CO_2 anesthesia when necessary.

Wing Measurements

Both wings were removed from each fly and fixed in DPX under coverslips on microscope slides with the dorsal side up. Bitmap images were captured with a video camera (Sony CCD-Iris, Tokyo, Japan) connected to a PC computer with MGI VideoWave software (Sonic Solutions, Novato, CA) and mounted on a compound microscope (Zeiss Axioskop, Jena, Germany), using a 2.5× objective. Calibration of the optical system was checked at each session. To quantify and minimize measurement error all wings were digitized two times at different sessions by one of us (C. Rego) as follows: images of both the left and right wings were captured during a given session and after an entire round on all individuals the same process was repeated. A similar procedure was also used to record the *x*- and *y*-coordinates of 14 morphological landmarks (i.e., labeled geometric points located at the intersections of wing veins or at sites where veins reach the wing margin; Fig. 1) by using the Fly Wing 15Lmk plug-in (kindly provided by C. P. Klingenberg, University of Manchester, U.K.) implemented in ImageJ 1.33u software (http://rsb.info.nih.gov/ij/). The process we used guaranteed that the observer was blind with respect to the results from previous measurements.

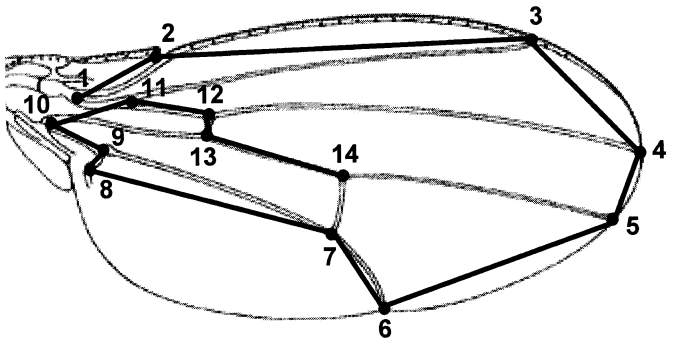

FIG. 1. Right wing of *Drosophila* showing the 14 landmarks used in this work. The landmarks have been connected by a thick line to enhance visibility of the resulting wing shape profile.

Analysis of Wing Size and Shape

This study employs geometric morphometrics analyses that precisely separate morphological variation (i.e., variation in form) into size and shape components (Bookstein 1991, 1996; Dryden and Mardia 1998; Adams et al. 2004; Zelditch et al. 2004). Size is a one-dimensional trait and the measure we used was centroid size (CS), computed here in a normalized form as the square root of the sum of the 28 squared Euclidian distances (*x* and *y* directions separated) of the 14 landmarks to the centroid (center of gravity) divided by the square root of the number of landmarks (Dryden and Mardia 1998, p. 24). Individual size is therefore represented by four scalars, one for each side and session.

Procrustes superimposition provides a convenient method to characterize shape variation. However, it is important to realize that in geometric morphometrics shape is defined as a character of the entire landmark configuration and, therefore, it is necessarily multivariate. In addition, it should be remembered that the removal of size, position, and orientation after Procrustes superimposition reduces the dimensional space to $2p - 4$, where p is the number of landmarks (e.g., Goodall 1991). Thus, for the present study of 14 landmarks, with two coordinates each, the shape dimension is 24. Because the dataset included both left and right wings (i.e., we are dealing with matching symmetry; see Mardia et al. 2000; Klingenberg et al. 2002), the landmark configurations of left wings were reflected to their mirror images by changing the sign of all *x*-coordinates (see also Klingenberg and McIntyre 1998). Then, all individuals of all configurations combined were superimposed simultaneously by a generalized least-squares Procrustes fit. The final iteration to minimize the sum of the squared distances between the landmarks of all wings in the sample was done without additional scaling and, consequently, we performed a partial Procrustes fit according to Dryden and Mardia (1998; see also Rohlf 1999). Given the small amounts of shape variation in this analysis, rescaling the coordinates of each configuration by the scaling option

$1/\cos(\rho)$ (see Rohlf 1999) would have negligible effects on the results. Scatterplots of superimposed Procrustes coordinates were visually inspected for gross outliers (e.g., mislabeling of landmarks) by using the plot subroutine in the MATLAB algebra program environment (ver. 7.0.4, MathWorks 2005a).

Asymmetry Analyses

Univariate analyses

Right-left asymmetries were calculated for CS (in ln mm) using the conventional two-way mixed model ANOVA with sides as fixed factors and individuals as random factors (Leamy 1984; Palmer and Strobeck 1986). Ln-transforming all data removes linear size dependence for FA (e.g., Palmer and Strobeck 2003). The individual effect stands for phenotypic variation, side is for DA and tests whether the signed differences between the right and left wings (designated as $[R - L]$) has a mean of zero. The interaction term is a measure of FA (the variation in $[R - L]$ among individuals) provided that there is no genetic variation for DA (see Santos 2001, 2002). Finally, the error term gives an estimate of the measurement error.

Whenever feasible, up to four randomly sampled *DmDs* hybrid females and males emerged from each vial were used for morphometric analyses. Because not all parental females from the *D. madeirensis–D. subobscura* hybrid half-sib breeding design produced enough offspring of both sexes (see above), the final dataset was an unbalanced nested classification. Thus, a total of 172 hybrid females were measured from 40 sires and 86 dams, and a total of 203 hybrid males from 47 sires and 98 dams. Only 26 dams and 44 sires were used for morphometric analysis. The relatively low number of dams used was basically due to badly damaged wings in most of them.

The ANOVA model of analysis for the hybrid half-sib breeding design to estimate causal variance components was:

$$y_{ijkl} = \mu + \alpha_i + \delta_{j(i)} + b_{k(ij)} + e_{ijkl}, \qquad (1)$$

where μ is the overall grand mean, α_i is the random effect of the *i*th male (sire), $\delta_{j(i)}$ is the random effect of the *j*th female (dam) within the sire *i*, $b_{k(ij)}$ is the between-fly effect within the sire *i* and dam *j*, and e_{ijkl} is the residual error associated with the ln(CS) of the *l*th measurement in the *ijk*th offspring. The error variance includes side, individual × side interaction, and measurement error effects that can be further partitioned out. In particular, the individual × side interaction will allow estimating causal variance components related with FA/DA (see details below).

A practical note is in order here. Restricted maximum likelihood (REML) has emerged as the preferred method for estimating variance components because it uses all the available information in the pedigrees, it can easily accommodate unbalanced designs, and software packages such as VCE5 are freely available (http://vce.tzv.fal.de/index.pl/getvce). However, the among-sire variation in the hybrid half-sib breeding design does not reflect the additive genetic variance in the standard sense. As stated by Falconer and Mackay (1996, ch. 19) the expression of a trait in two different environments (i.e., pure species and hybrid genetic backgrounds) can be considered as two distinct characters that may be genetically correlated. Furthermore, the segregating genes in *D. subobscura* that interact epistatically with fixed differences in *D. madeirensis* will appear as among-sire variation. Some traits that may appear in *DmDs* hybrids tend to be deleterious (e.g., deformed head shape, abnormalities of abdominal tergites, and male sterility; see introduction), which is clear evidence of epistatic gene action because these genes cause abnormalities only in the interspecific and not in the intraspecific crosses. The Bateson-Dobzhansky-Muller model (Gavrilets 2004), in which the accumulation of complementary substitutions results in hybrid problems, provides a simple scenario for those observations. The X chromosome of *D. madeirensis* seems to be a major determinant of phenotypic abnormalities in the mixed genetic background, while the Y chromosome plays an important role in male sterility (Khadem and Krimbas 1993). These limitations do not, in any case, impose important constraints in our analyses.

The convenience for not averaging the individual ln(CS) measurements in model (1) is because it can be straightforwardly subsumed in the mixed model, two-way ANOVA for the study of right-left asymmetries (Santos 2001, 2002). Permutation tests (particularly necessary with unequal sample sizes; see Edgington 1995) can also be easily performed to test all random components. For the three-level nested ANOVA, model randomization is a three-stage process: (1) random permutations among offspring within sire and dam for the between-fly *F*-statistics; (2) random permutations among offspring and dam within sire for the dam in sire *F*-statistics; and (3) random permutations among offspring, dam, and sire for the among-sire *F*-statistics. Each test used 10,000 random permutations of the observations. Finally, delete-one-sire-family jackknife data resampling was carried out as a robust test to estimate the genetic components of variance (Knapp et al. 1989; Mitchell-Olds and Bergelson 1990; Sokal and Rohlf 1995). Thus, a total of $n_f = 40$ pseudovalues for the hybrid females and $n_m = 47$ for the hybrid males were obtained by dropping, in turn, each sires' family and calculating:

$$\phi_i = N\hat{\Theta}_N - (N - 1)\hat{\Theta}_{N-1,i} \qquad (2)$$

(caret denotes an estimator of), where ϕ_i is the *i*th pseudovalue, $\hat{\Theta}_N$ is the corresponding variance estimate using all *N* families, and $\hat{\Theta}_{N-1,i}$ is that estimate calculated by dropping the *i*th family alone. Causal components of variance (i.e., σ^2_α, σ^2_δ and σ^2_b) in model (1) were estimated following Sokal and Rohlf (1995, pp. 294–299). The jackknife estimate is the average of ϕ_i, and its standard error is given by

$$\text{SE} = \sqrt{\frac{\sum_{i=1}^{i=N} (\phi_i - \bar{\phi})^2}{N(N-1)}}. \qquad (3)$$

Approximate 95% jackknife confidence intervals were obtained as $\bar{\phi} \pm 2$ SE. Dominance variance and/or maternal effects (together with the 95% jackknife confidence intervals) were estimated as $\hat{\sigma}^2_{dm} = \hat{\sigma}^2_\delta - \hat{\sigma}^2_\alpha$ (dominance here refers to an allele's effect on a hybrid genetic background).

The residual sum of squares (model 1) was partitioned into sides, individual × side, and measurement sum of squares.

TABLE 1. Analyses of variance for centroid size (in ln mm). The analyses were done on the average of both wings and measurements for each individual (data plotted in Fig. 2). Results from Scheffé post hoc tests are also shown. Species: *Dm*, *Drosophila madeirensis*; *Ds*, *D. subobscura*; and *DmDs* or *DsDm*, the corresponding hybrids with the parental female species indicated first.

Sample	Source	df	SS	MS	*F*	*P*
Females	species	2	0.08085	0.04042	27.15	<0.001
	error	244	0.36330	0.00149		
	total	246	0.44415			
			DmDs ≥ *Dm* > *Ds*			
			(*P* = 0.051)			
Males	species	3	0.26766	0.08922	92.40	<0.001
	error	337	0.32539	0.00097		
	total	340	0.59305			
			Dm = *DmDs* > *Ds* = *DsDm*			
		(*P* = 0.333)		(*P* = 0.769)		

To test for hybridization effects on developmental stability some conditions must be met: estimates of FA should not be inflated by a nongenetic systematic bias due to DA variation and/or heritable differences in bilateral asymmetries. Thus, genetic and environmental variation in DA biases the analyses of FA because the variance component from the individual × side interaction term would combine true FA plus genetic and environmental components of DA (Santos 2001, 2002; Stige et al. 2006). Under our standardized laboratory conditions, we expect the nongenetic variance in DA to be about the same in hybrids and pure species. Given our half-sib hybrid design, the individual × side sum of squares was further partitioned into sires, dams, and within-fly components to test for genetic variation in DA. Permutation tests were performed by using DA as the dependent variable in a two-level nested ANOVA model with sires and dams in sires as random factors. As before, each test used 10,000 random permutations of the observations.

Multivariate analyses

It is fairly straightforward to extend all the preceding ANOVA methodology to the multivariate (MANOVA) situation required for shape analyses because all effects are computed from averages or contrasts in the same shape space (Klingenberg et al. 2002; Santos et al. 2005). Because four degrees of freedom are lost in the Procrustes procedure, sums of squares and cross-products (SSCP) matrices are not full-ranked, and the degrees of freedom need to be adjusted. There are three alternative ways of avoiding these difficulties (Dryden and Mardia 1998; Klingenberg et al. 2002): (1) to omit, after Procrustes superimposition of the complete configurations, the coordinates of any two landmarks; (2) to retain 24 PC (principal components; Jolliffe 1986) scores from the covariance matrix of the dataset; and (3) to slightly modify the multivariate statistics by using the Moore-Penrose generalized inverse of the SSCP matrices so they can tolerate singular matrices and compute the product of nonzero ei-

TABLE 2. Asymmetry of overall wing size (CS in ln mm) in the two parental *Drosophila* species and the hybrid males (*DsDm*) from the mass crosses ♀♀ *D. subobscura* × ♂♂ *D. madeirensis*. The denominator mean square (MS) to calculate *F*-values for the main random (individual) and fixed (side) effects is that for the interaction term (all values ×10⁴). The values for the estimated $\sigma^2_{I\times S}$ variance component (FA) are also given.

Sample	Source	df	SS	MS	*F*	$\sigma^2_{I\times S}$
♀♀ *D. madeirensis*	individuals (I)	25	2716.3775	108.6551	56.33***	
	sides (S)	1	0.5914	0.5914	0.31	
	I × S	25	48.2100	1.9288	3.74***	0.69974
	measurement	52	27.0521	0.5293		
♀♀ *D. subobscura*	individuals	48	1157.3884	24.1123	39.36***	
	sides	1	0.9297	0.9297	1.52	
	I × S	48	29.4067	0.6126	3.81***	0.22588
	measurement	98	15.7568	0.1608		
♂♂ *D. madeirensis*	individuals	45	1623.6810	36.0818	72.33***	
	sides	1	1.9198	1.9198	3.85†	
	I × S	45	22.4482	0.4988	2.35***	0.14310
	measurement	92	19.5638	0.2126		
♂♂ *D. subobscura*	individuals	43	681.8630	15.8573	45.00***	
	sides	1	0.0111	0.0111	0.03	
	I × S	43	15.1546	0.3524	1.95**	0.08566
	measurement	88	15.9324	0.1810		
♂♂ *DsDm*	individuals	47	838.4231	17.8388	36.70***	
	sides	1	0.0584	0.0584	0.12	
	I × S	47	22.8471	0.4861	13.95***	0.22564
	measurement	96	3.3444	0.0348		

† $0.10 > P > 0.05$; ** $P < 0.01$; *** $P < 0.001$.

TABLE 3. Asymmetry of centroid size (CS, in ln mm) and ANOVA estimates of causal variance components of size (σ^2[CS]) and DA of size (σ^2 [DA_{CS}]), in interspecific hybrids raised from the ♀♀ *Drosophila madeirensis* × ♂ *D. subobscura* hybrid half-sib breeding design (all values × 10^4). Average CS for right (*R*) and left (*L*) wings: females $R = 1.0017 \pm 0.0019$ mm, $L = 1.0174 \pm 0.0027$; males $R = 0.9278 \pm 0.0016$, $L = 0.9301 \pm 0.0016$.

Sample	Source	df	SS	MS	Variance component	Direct estimate	Jackknife estimate	Lower and upper 95% limits	
Females	individuals (I)	171	10,566.233	61.7908***	σ^2_I(CS)	11.98131	12.10814	3.89382	20.32245
	sires	39	3427.572	87.8865	σ^2_α(CS)	0.72470	0.70104	−1.15053	2.55261
	dams	46	3411.673	74.1668***	σ^2_δ(CS)	3.93526	4.00848	−0.71771	8.73467
	dominance				σ^2_{dm}(CS)	3.21056	3.30744	−2.61302	9.22790
	between-fly	86	3726.988	43.3371***	σ^2_b(CS)	7.36787	7.39872	1.11872	13.67872
	sides (S)	1	385.716	385.7156***					
	I × S	171	2371.015	13.8656***	$\sigma^2_{I\times S}$(CS)	6.03802	6.04816	3.50304	8.59329
	sires	39	1035.533	26.5521*	σ^2_α(DA_{CS})	3.01795	2.98972	−0.76923	6.74867
	dams	46	617.693	13.4281	σ^2_δ(DA_{CS})	2.59462	2.55473	−1.72898	6.83843
	dominance				σ^2_{dm}(DA_{CS})	−0.42332	−0.43499	−7.36937	6.49939
	within-fly	86	717.790	8.3464***	σ^2_w(DA_{CS})	3.27843	3.27575	1.16149	5.39000
	measurement	344	615.600	1.7895	σ^2_{me}(CS)	1.78954	1.78600	0.69069	2.88132
	total	687	13,938.564						
Males	individuals (I)	202	9874.800	48.8851***	σ^2_I(CS)	12.03455	12.09824	7.97583	16.22066
	sires	46	3666.719	79.7113	σ^2_α(CS)	0.82266	0.80787	−1.75254	3.36828
	dams	51	3208.970	62.9210***	σ^2_δ(CS)	4.29857	4.32256	−0.28087	8.92600
	dominance				σ^2_{dm}(CS)	3.47590	3.51469	−3.20735	10.23673
	between-fly	105	2999.111	28.5630***	σ^2_b(CS)	6.95401	6.96771	4.85392	9.08150
	sides (S)	1	13.075	13.0749***					
	I × S	202	150.877	0.7469***	$\sigma^2_{I\times S}$(CS)	0.27620	0.27640	0.18200	0.37081
	sires	46	42.821	0.9309†	σ^2_α(DA_{CS})	0.07936	0.07840	−0.02941	0.18620
	dams	51	30.544	0.5989	σ^2_δ(DA_{CS})	0.06798	0.06908	−0.09412	0.23227
	dominance				σ^2_{dm}(DA_{CS})	−0.01137	−0.00932	−0.15367	0.13503
	within-fly	105	77.149	0.7348***	σ^2_w(DA_{CS})	0.27012	0.27080	0.14960	0.39200
	measurement	406	78.976	0.1945	σ^2_{me}(CS)	0.19452	0.19454	0.16187	0.22721
	total	811	10,117.727						

† $0.10 > P > 0.05$; * $P < 0.05$; *** $P < 0.001$. The tests for the causal variance components are based on 10,000 random permutations (see text for details).

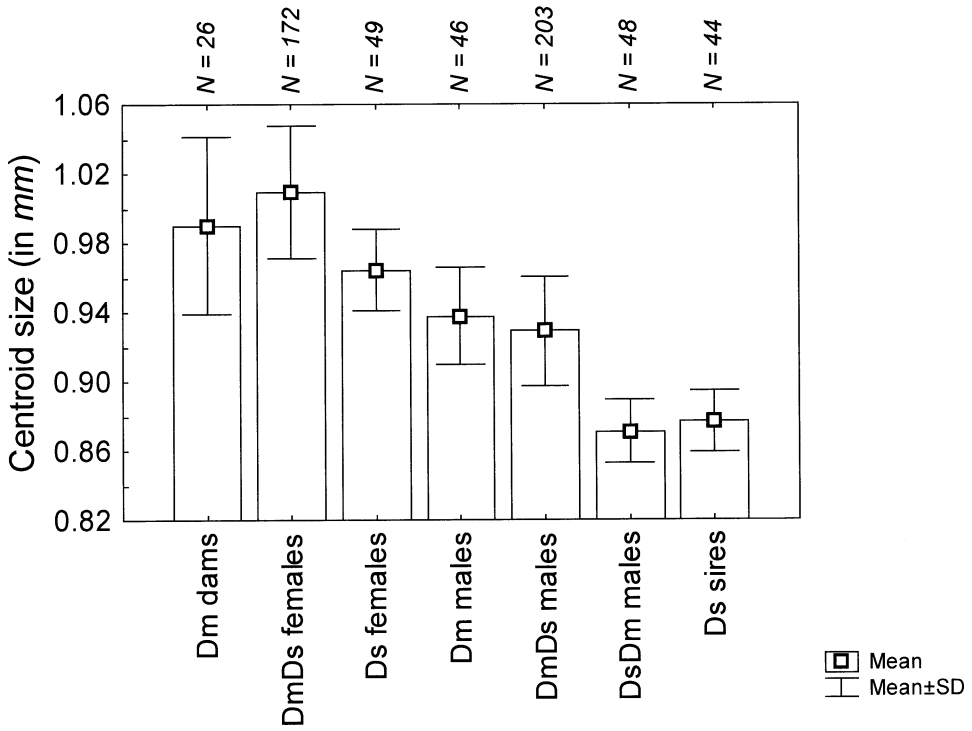

FIG. 2. Average centroid size (estimated here in a normalized form; see Dryden and Mardia 1998, p. 24) of the two pure *Drosophila* species and their F_1 hybrids. *Dm*, *D. madeirensis*; *Ds*, *D. subobscura*; and *DmDs* or *DsDm*, corresponding hybrids with the parental female species indicated first. The top axis gives the number of individuals measured for each sample.

genvalues instead of the determinant of SSCP matrices. We have employed here the second scheme by using the ''princomp'' subroutine in the Statistics Toolbox (ver. 5.0.2, MathWorks 2005b) of the MATLAB algebra program environment (ver. 7.0.4, MathWorks 2005a). The degrees of freedom (df 1) in the MANOVAs are simply the corresponding degrees of freedom in the ANOVAs for centroid size times the number of PC scores retained in each sample.

Because allometry has been shown to occur in *Drosophila* wing shape (although usually accounting for a small part of shape variation; see Debat et al. 2003) and we are comparing two different *Drosophila* species and their hybrids, the allometric component of shape variation was removed by using ln(CS) as a covariate in the MANOVAs. The SSCP matrices were divided by the appropriate degrees of freedom, and effects were separated according to the expected mean squares in the ANOVA by subtracting the interaction covariance (VCV) matrix from the interindividual VCV matrix and the error VCV matrix from the interaction one. The overall nonallometric covariation in wing shape (individuals effect) was further decomposed into causal components (sires, dams, and between-fly), and the covariation in right-left asymmetries (individual × sides interaction effect) into causal components attributable to wing shape DA.

Visualizing Patterns of Shape Variation

VCV matrices were constructed separately for females and males after removing the allometric component. Principal component analyses (Jolliffe 1986) of the VCV matrices were performed for each source of variation to describe the landmark displacements corresponding to each emerging PC and to test for the congruence of these displacements between effects (see Klingenberg and McIntyre 1998; Debat et al. 2000; Santos et al. 2005). Delete-one-sire-family jackknife data resampling was also used to estimate VCV matrices, but results (not shown) were qualitatively identical to those obtained with the direct estimates and only the latter will be used here.

The between-fly VCV_b matrix (i.e., the within-family residuals) includes a fraction of the genetic variance and the entire environmental component (see Becker 1984, p. 56), which can be roughly estimated as $VCV_{bE} = VCV_b - (VCV_\alpha + VCV_\delta)$. It should be noted, however, that dominance, additive × additive, and additive × dominance (co)variances are also included in the between-fly VCV_b matrix, which may be important in the hybrid half-sib breeding design because interactions in the Bateson-Dobzhansky-Muller model involve both dominance and epistasis (Turelli and Orr 1995, 2000). Hence, VCV_{bE} would be biased as it includes a fraction of those components.

Matrix correlations were computed from the upper triangular part (diagonal entries were included) because VCV matrices are symmetrical, and statistical significance was assessed using permutation tests designed to maintain the intrinsic association between landmark coordinates (i.e., by shuffling together the *x*- and *y*-coordinates of a particular landmark; Klingenberg and McIntyre 1998; Debat et al. 2000; Santos et al. 2005); otherwise the null hypothesis would imply the complete absence of all geometric structure. The permutation procedure was carried out 10,000 times. Correlative

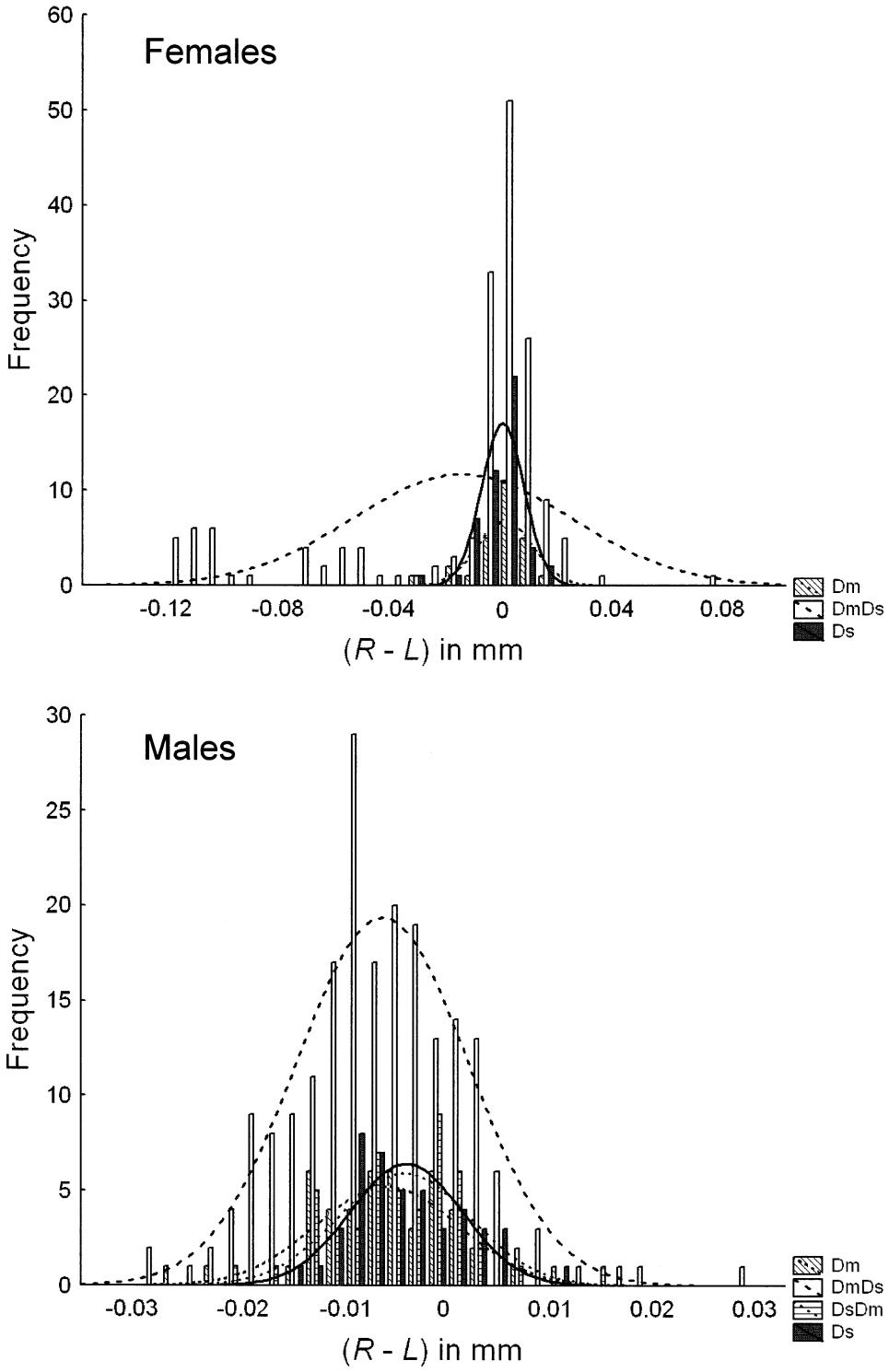

FIG. 3. Distributions of signed right − left ($R - L$) asymmetries for centroid size in females (upper panel) of *D. madeirensis* (*Dm*; mean ± SE: −0.00157 ± 0.00232; SD: 0.01181), *D. subobscura* (*Ds*: −0.00135 ± 0.00108; 0.00754), and interspecific hybrids (*DmDs*: −0.01573 ± 0.00295; 0.03867); and in males (lower panel) of *Dm* (−0.00190 ± 0.00098; 0.00664), *Ds* (−0.00012 ± 0.00078; 0.00517), and interspecific hybrids *DmDs* (−0.00232 ± 0.00055; 0.00790) and *DsDm* (−0.00031 ± 0.00088; 0.00611). Only *DmDs* female hybrids were more asymmetric (i.e., higher DA) than pure species, which did not differ between them (see text for details). To enhance visibility the normal distributions fitting the datasets are also shown (notice the different scales in females and males).

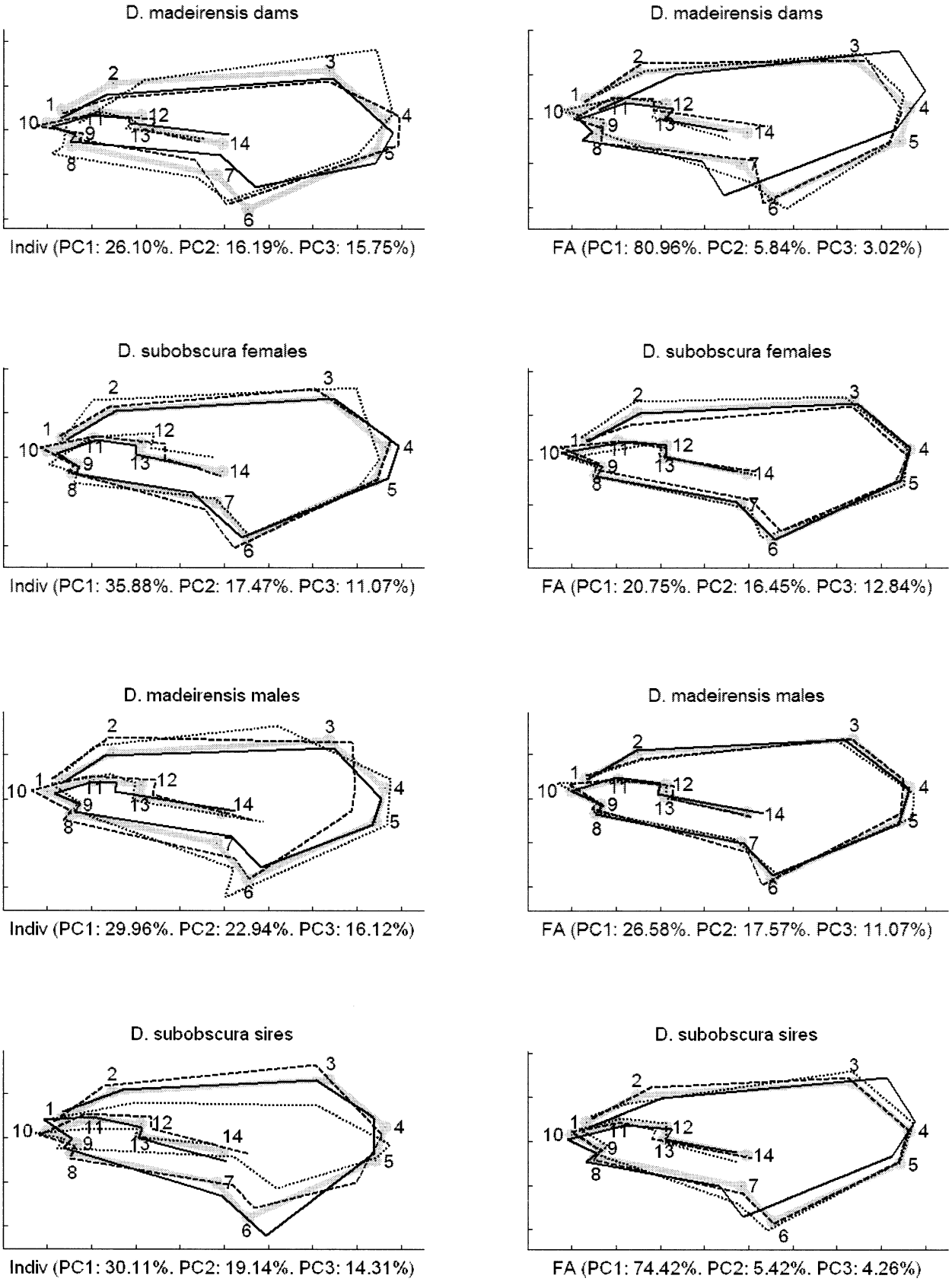

FIG. 4. Joint variation along the three first principal components (PCs) for individual and for fluctuating asymmetry in the pure species (*Drosophila madeirensis* and *D. subobscura*) used in this study. The PCAs for individual variability used the nonallometric among-

patterns of whole shape variation are difficult to interpret: a significant correlation would suggest a real congruence, but a weak congruence does not imply a significant correlation.

A second test examined the congruence of the landmark displacements corresponding to each emergent PC for the different effects. Because the PCs correspond to directions in the multivariate shape space, correlations can be obtained by angular comparisons of component vectors. Statistical significance was assessed by comparing the observed values to a null distribution of absolute angles between 100,000 pairs of 24-dimensional random vectors in a unit sphere. The 0.1% and 0.001% quantiles of the resulting distribution were 51.8° and 42.3°, respectively.

Computer Software for Statistical Analysis

The computer programs used for statistical data analyses were MATLAB algebra program environment (ver. 7.0.4, MathWorks 2005a) together with the collection of tools supplied by the Statistics Toolbox (ver. 5.0.2, MathWorks 2005b), and the statistical software packages STATISTICA version 6 (2003) and SPSS version 13 (2004). Some helpful functions in morphometrics from the MATLAB toolbox Res6 developed by R. E. Strauss (available at http://www.biol.ttu.edu/Strauss/Matlab/matlab.htm) were also used.

RESULTS

Variation and Asymmetry in Size

The averages for centroid size in the parental species and their hybrids are plotted in Figure 2. *Drosophila madeirensis* is clearly larger than *D. subobscura*, and the size of the interspecific hybrids is about the same as that of the matching sex from the maternal species (Fig. 2, Table 1). The analyses of size asymmetries indicated that there was significant FA (individual × side interaction effect) in all cases (Tables 2, 3), as well as subtle but significant DA (side effect) in the *DmDs* interspecific hybrids (see the summary statistics for right and left wings at the bottom of Table 3). We cannot conclude that DA was generally absent in the parental species because of the relatively low statistical power to detect it; however, DA was close to zero in both pure species (see Fig. 3). Interestingly though, left wings were consistently larger than the right ones in all cases, which agrees with previous findings in *D. subobscura* (Fernández Iriarte et al. 2003; Santos et al. 2005). There was, in addition, some indication of additive genetic variation for DA (i.e., σ^2_α [DA_{CS}]) as judged from the permutation tests (Table 3). Finally, the contrasts for centroid size DA comparing *DmDs* versus pure species indicated that only female hybrids were more asymmetric (females $F_{1,244} = 9.19$, $P = 0.003$; males $F_{1,337} = 2.06$, $P = 0.152$). (We elaborate on this point below.)

We found nonsignificant among-sire variation for centroid size in the hybrid half-sib breeding design (Table 3). It is quite unlikely that this could be due to a lack of additive genetic variation for body size in the *D. subobscura* base stock because a relatively large number of wild flies were used as founders, and the population was kept at a high number of breeding adults since its foundation. In addition, *Drosophila* size traits typically have intermediate heritabilities in the laboratory (Roff and Mousseau 1987). However, significant among-dam variation was observed from the permutation tests, although the 95% jackknife confidence intervals included zero. Together with the results plotted in Figure 2, it seems that maternal effects are pivotal in determining hybrids' size but, as for the among-dam variation, dominance and/or maternal effects were not statistically significant when judged from the jackknife procedure.

Figure 3 shows the distributions of signed $(\bar{R} - \bar{L})$ asymmetries from both parental species and the interspecific hybrids. A substantial increase in wing size asymmetry was mainly detected for females where both the average and variance of DA were significantly greater as compared to pure species, which obviously translates into a higher FA in hybrid females that can be readily seen from the estimates of the interaction terms (6.03802 for *DmDs* vs. 0.69974 for *D. madeirensis* and 0.22588 for *D. subobscura*; Tables 2, 3). However, a substantial fraction of the total variation in centroid size DA in *DmDs* hybrid females seems to be due to family differences in average DA because we have detected significant among-sire variation from the permutation tests (Table 3). A more accurate (but still biased) estimate of FA would be provided by the within-fly source of variation in Table 3 ($\hat{\sigma}^2_w[DA_{CS}] = 3.27843$). Yet, this estimate also includes a fraction of the additive genetic variance (one-half from standard nested full-sib, half-sib mating designs; see Lynch and Walsh 1998, p. 572) that should be removed by, for example, subtracting twice the among-sire component to obtain an unbiased estimate of (genuine) FA: $\hat{\sigma}^2_{FA} = \hat{\sigma}^2_w(DA_{CS}) - 2\hat{\sigma}^2_\alpha(DA_{CS})$.

The problem here, however, is that the among-sire (among-dam) variation in the hybrid half-sib breeding design does not reflect the additive genetic variance in the standard sense. The segregating genes in *D. subobscura* that interact epistatically with fixed differences in *D. madeirensis* will appear as among-sire variation and, conversely, those segregating in *D. madeirensis* will appear as among-dam variation. This implies, in turn, that unlike the standard situation with pure species the fraction of additive genetic variance coming from sires is not necessarily the same as the fraction coming from dams. In any case, this does not substantially change the way to appropriately correct the within-fly source of variation to obtain an unbiased estimate of FA, which is better obtained here (assuming that the majority of DA genetic variation is additive) as $\hat{\sigma}^2_{FA} = \hat{\sigma}^2_w(DA_{CS}) - \hat{\sigma}^2_\alpha(DA_{CS}) - \hat{\sigma}^2_\delta(DA_{CS})$ (i.e., by subtracting the sum of sire and dam effects to the within-fly variation). This renders $\hat{\sigma}^2_{FA} = -2.33414$ (jackknife es-

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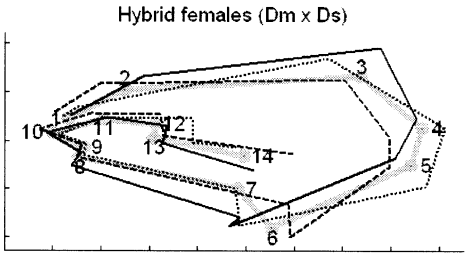
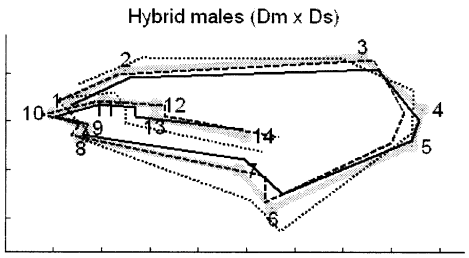
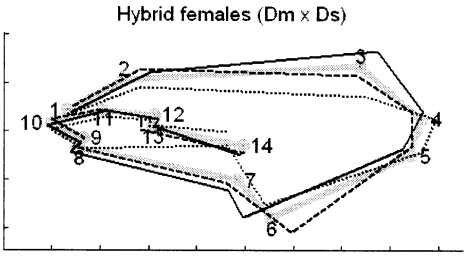
individual covariance matrix corrected for the intraindividual variation and measurement error, and the PCAs for fluctuating asymmetry used the nonallometric individual × side covariance matrix corrected for measurement error. The thick gray line plots the average shape for each sample; the solid line the PC1, the dashed line the PC2, and the dotted line the PC3 coefficients. Percentages of total shape variation explained by the PCs for the corresponding covariance matrices are also given. Landmark positions are indicated by numbers.



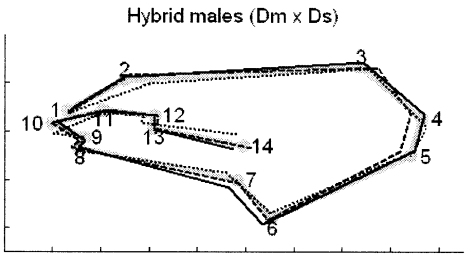
Indiv (PC1: 35.71%. PC2: 22.10%. PC3: 10.12%)



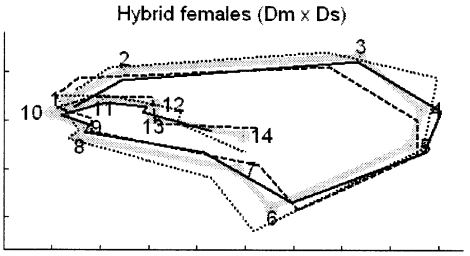
Indiv (PC1: 24.23%. PC2: 16.71%. PC3:12.59%)



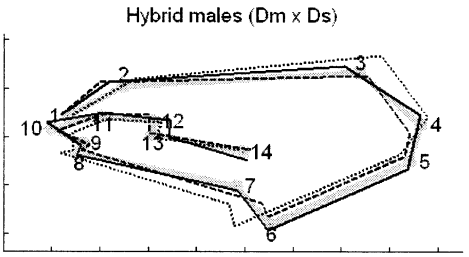
Sire (PC1: 47.42%. PC2: 12.93%. PC3: 11.57%)



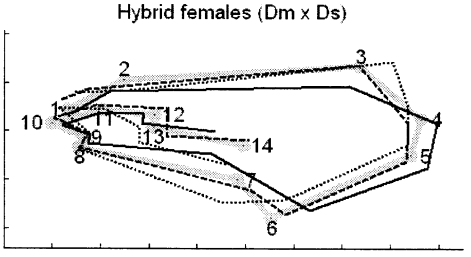
Sire (PC1: 22.96%. PC2: 15.12%. PC3: 12.00%)



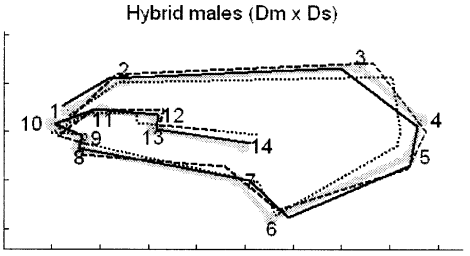
Dominance/maternal (PC1:39.01%. PC2:20.89%. PC3:10.64%)



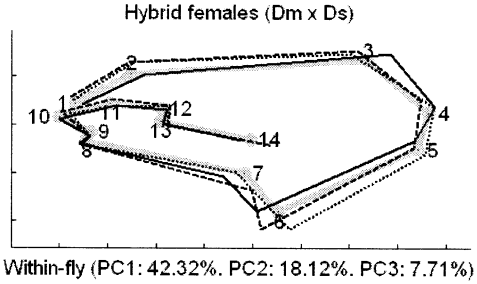
Dominance/maternal (PC1:29.53%. PC2: 17.57%. PC3:13.70%)



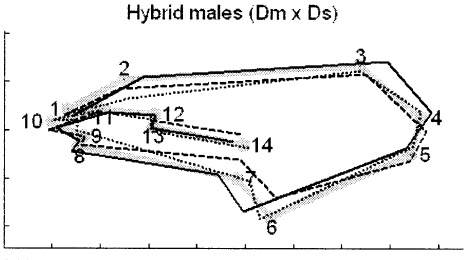
Environmental (PC1: 54.16%. PC2: 10.30%. PC3: 7.89%)



Environmental (PC1: 26.04%. PC2: 18.40%. PC3: 12.11%)



Within-fly (PC1: 42.32%. PC2: 18.12%. PC3: 7.71%)



Within-fly (PC1: 56.68%. PC2: 11.96%. PC3: 5.52%)

timate: −2.26871; 95% confidence interval: −7.32231, 2.78490) for *DmDs* hybrid females, which clearly indicates that there is much more to hybrid bilateral asymmetry than just developmental instability. This figure is obviously unrealistic (there is nothing in the ANOVA method of estimation that will prevent a negative variance estimate), as the random noise involved in the development of bilateral morphological structures will invariably result in FA greater than zero. However, the conclusion that the increase in wing size asymmetry observed in female hybrids (Fig. 3) cannot be attributed to an increase of intraindividual variation due (only) to random developmental noise seems sound. We make a caveat, however: when comparing the unbiased FA estimate in female hybrids with those obtained from the interaction terms in the parental species, we are assuming that these terms provided FA estimates that are not inflated by genetic variation in DA. This assumption is sustained by some previous results with isochromosomal lines of *D. subobscura* showing that the estimate of the interaction term for centroid size was about the same to that for the within-fly source of variation, as expected if there is little or no genetic variance for DA within this species (Santos et al. 2005, table 1).

Variation and Asymmetry in Shape

Using species and sex as categorical predictors and ln(CS) as a covariate, a two-way MANCOVA analysis on the 24 PC scores from the Procrustes coordinates as dependent variables detected highly statistically significant differences for all effects (results not shown).

The two-way MANCOVA analyses to quantify nonallometric (i.e., ln[CS] was used as a covariate) shape variation within samples detected a highly significant individual × side interaction effect in all cases (FA), but the side effect (DA) was only statistical significant for *DmDs* hybrids (both females and males) and *D. subobscura* males (results not shown). It seems, therefore, that wing shape DA cannot be X-linked because *DmDs* male hybrids did not receive an X chromosome from its *D. subobscura* sire. Incidentally, genetic variation for DA unlinked to the X chromosome has been previously uncovered for *D. subobscura* (Santos et al. 2005). Finally, the contrasts for wing shape DA between *DmDs* versus pure species were statistically significant (females Wilks' $\lambda = 0.836$, $P = 0.030$; males Wilks' $\lambda = 0.816$, $P = 0.001$), clearly indicating that DA was indeed higher in those hybrids.

To test for hybridization effects on wing shape FA, we relied on the traces of the individual × side SSCP matrices divided by the corresponding degrees of freedom because the trace of those matrices is straightforwardly related to the sum of $\mathrm{Var}(\bar{R} - \bar{L})$ (index FA4 in Palmer 1994) for each *x*- and *y*-coordinate of the corresponding aligned configurations divided by the shape dimension, in dealing with random perturbations in development only the magnitude of those perturbations is generally of interest, and no genetic variation for wing shape DA was detected in our *DmDs* samples (results not shown; we obviously cannot test here for putative DA genetic variation segregating within species). Therefore, permutation tests (including ln[CS] as a covariate) were easily carried out by randomly allocating individuals to groups and comparing the observed ratio of matrix traces with the distribution of ratios obtained from 10,000 random permutations. The results (not shown) can be easily summarized as follows: (1) the phenotypic anomalies observed in F_1 males according to the direction of cross (see introduction) are somewhat reproduced here since (nonallometric) wing shape FA in *DmDs* males was substantially larger than in *DsDm* males; and (2) we cannot conclude that hybridization per se increases FA since hybrids from both sexes display similar or even lower levels of FA than one of the parental species.

Visualizing Shape Variation

VCV matrices were constructed separately for females and males from the two-way MANCOVA analyses to quantify nonallometric shape variation within samples.

Pure species

Figure 4 shows the variation for the individual and FA sources for pure species along the first three PCs. Visual inspection suggests modest congruence in the magnitude and direction of shape changes between species, and permutation tests indicated that matrix correlations (MCs) were relatively low for both individual (females: MC = 0.4777, $P = 0.0652$; males: MC = 0.3887, $P = 0.0234$) and FA (females: MC = 0.3304, $P = 0.0672$; males: MC = 0.3486, $P = 0.0511$) components. However, we use here *P*-values as relative measures for similarity of trends (see Berger 2003) and, accordingly, we do not correct for multiple testing.

The angles between the three dominant PCs also reflect the poor correspondence between species. Thus, for the individual variation the minimum observed angle for females was between the PC1 of *D. madeirensis* and the PC2 of *D. subobscura* ($\alpha = 49.2°$) and for males between the two PC1 of both species ($\alpha = 60.7°$). Similar low associations were also found between species FAs: the minimum angle was between the PC1s of males ($\alpha = 60.2°$).

Matrix correlations for the shape changes of individual variation and FA are given in Table 4. Within pure species, MCs were only sizeable for *D. subobscura* females and *D. madeirensis* males (results were qualitatively similar for observed angles; not shown). However, patterns of covariation between FA and measurement error were highly concordant in all samples. When considered together, these results suggest a weak relationship between (overall) canalization (i.e.,

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FIG. 5. Joint variation along the three first principal components (PCs) for the different effects estimated from the *D. madeirensis–D. subobscura* hybrid half-sib breeding design (allometry was removed by including ln[CS] as a covariate). The thick gray line plots the average shape for each sample; the solid line the PC1, the dashed line the PC2, and the dotted line the PC3 coefficients. Percentages of total shape variation explained by the PCs for the corresponding covariance matrices are also given. Landmark positions are indicated by numbers.

TABLE 4. Matrix correlations (MC) between VCV matrices of landmarks displacements within groups (the first column refers to the matrices being compared). For the pure species and *DsDm* male hybrids VCV matrices are only available for the random effects in the MANCOVAs for the study of nonallometric right-left asymmetries. *DmDs* or *DsDm* stand for the corresponding hybrids with the parental female species indicated first.

Effect	♀♀ *DmDs*[1]		♀♀ *D. madeirensis*		♀♀ *D. subobscura*		♂♂ *DmDs*[1]		♂♂ *D. madeirensis*		♂♂ *D. subobscura*		♂♂ *DsDm*	
	MC	$P_{perm.}$	MC	$P_{perm.}$	MC	$P_{perm.}$	MC	$P_{perm.}$	MC	$P_{perm.}$	MC	$P_{perm.}$	MC	$P_{perm.}$
Individual/FA	0.7780	0.0001	−0.1189	0.5681	0.5261	0.0120	0.0303	0.4621	0.6872	0.0001	0.0724	0.3401	0.5857	0.0217
Sire/FA	0.6644	0.0542					0.3163	0.1556						
Dam/FA	0.3822	0.0509					0.2066	0.2166						
Dominance/FA	−0.0128	0.4931					0.0477	0.3987						
Environment/dam	−0.8954	0.9999					−0.4570	0.9999						
Environment/FA	−0.3272	0.9916					−0.5316	0.7998						
Individual/error	0.6767	0.0119	−0.0671	0.5219	0.6471	0.0001	−0.1359	0.6624	0.4128	0.0107	0.1341	0.3437	0.2913	0.0110
FA/error	0.8571	0.0001	0.9388	0.0022	0.7562	0.0001	0.9492	0.0001	0.7840	0.0001	0.9659	0.0001	0.7721	0.0001

[1] Fluctuating asymmetry (FA) was estimated as the within-fly component in the MANCOVAs (i.e., the multivariate extension of the within-fly effect in the ANOVAs for centroid size in Table 3).

interindividual variation) and FA in these two closely related species, in agreement with previous findings in *D. subobscura* (Santos et al. 2005).

Interspecific hybrids

The *D. madeirensis–D. subobscura* hybrid half-sib breeding design allows us to investigate the relationship between measures of canalization (genetic and environmental) and developmental stability in interspecific hybrids. Figure 5 shows variation along the first three PCs for different sources derived from the MANCOVAs.

A relatively high congruence was found in the magnitude and direction of shape changes between *DmDs* and *DsDm* male hybrids for the individual (MC = 0.7271, $P = 0.0001$) and FA (MC = 0.7446, $P = 0.0001$) components. Matrix correlations within samples (Table 4) also suggest congruence between the individual variation and FA for *DmDs* females and *DsDm* males, which in the former case was mainly due to the sire's source of variation. However, no congruence at all was found between the environmental component of the interindividual variation and FA in either case, which can be due to the fact that our estimate of the environmental component is biased and does not accurately reflect the random variation among individuals (see above); namely, interindividual variation levels may also partly reflect genotypic differences. Similar conclusions were generally obtained when comparing the angles between the three dominant PCs (results not shown).

DISCUSSION

A major finding in this work was the large bilateral asymmetry for wing size in *D. madeirensis–D. subobscura* hybrid females, which was even obvious to the naked eye in some individuals. To better appreciate the effect of *D. madeirensis–D. subobscura* hybridization on wing size asymmetry of F_1 females it is worth saying that about 20% female hybrids have and FA2 index (i.e., mean $|R - L|/[(R + L)/2]$; Palmer 1994) larger than 5%. None of the females from the pure species had such a high figure. Actually, the average FA2 found in highly inbred *D. subobscura* females raised at the suboptimal temperature of 23°C (see Santos et al. 2005) was 0.81% (with a maximum of 2.72%). Flies with a FA2 index of about 5% are likely in the limit that allows proper flying, and a few of our hybrid *DmDs* females even have a FA2 higher than 10%. Such anomaly, however, cannot be plainly explained by an increase of developmental instability in hybrids since our unbiased estimate of FA (which is a proxy for random noise in developmental processes) was about the same or even lower than that for females in both parental species, but is clearly an indication of some aberrant developmental processes worth further investigation. As for other abnormal traits (head shape abnormality; see Khadem and Krimbas 1993) in addition to the extreme male-biased sex ratio in the *D. subobscura–D. madeirensis* reciprocal crosses, Haldane's rule is not obeyed because hybrid males were about as asymmetrical as the parental species.

Markow and Ricker (1991) suggested that the increase in asymmetry (FA) observed from hybridization will be a function of how closely related the parental taxa are, with FA

escalating with divergence time. In their study with the sibling species pair *D. melanogaster–D. simulans* (divergence time 2.3 ± 0.65 million years ago; Russo et al. 1995) the results were qualitatively similar to those found here (despite the fact that our species have apparently diverged much more recently: about 0.6–1 million years ago; Ramos-Onsis et al. 1998): female hybrids produced by *D. melanogaster* mothers had morphological abnormalities and increased FA, and very few females were produced when *D. simulans* were the mothers. Thus, female development was also clearly compromised in interspecific hybrids, but the roles of developmental noise and/or symmetry-breaking mechanisms unconnected to purely stochastic processes cannot be assessed from their data. Under the tenet that external right-left symmetry was the default state it was somewhat reasonable to assume that an increase in FA was mostly brought about by a parallel increase in developmental instability. However, this cannot be taken for granted, and the possibility of underlying genetic mechanisms altering DA (Markow and Ricker [1991] also found significant directional asymmetry for wing lengths) is a quite serious prospect. Somite formation is just the primary example of how bilateral symmetry is first established in the vertebrate embryo (Kawakami et al. 2005), and we already know that in *Drosophila* there is a developmental mechanism for the developmental asymmetry (Ligoxygakis et al. 2001). Divergence between species in regulatory pathways may contribute to hybrid disruptions, and it would be interesting to relate these findings with patterns of gene expression to identify those pathways. For instance, Ranz et al. (2004) found a higher number of underexpressed genes in *D. melanogaster–D. simulans* hybrid females, which could be related to their higher asymmetry/developmental problems.

Alibert and Auffray (2003, table 8.3) have summarized the relationship between developmental stability and hybridization, and concluded that most published works (71%) do show an increase of FA from crosses of different genera or species. But in our opinion we still lack a compelling answer to the chief question: Are developmental stability processes buffering the developmental noise that affects left and right sides of bilateral traits (what FA is really about) less successful in hybrids between those divergent lineages? Aside from statistical issues (see Palmer and Strobeck 2003), standard analyses of FA, namely, a straightforward application of the two-way ANOVA (or its MANOVA generalization) to test for individual × side interaction effects, does not provide any clue to the underlying causal mechanisms of bilateral asymmetry. The problem can be even more acute when working with interspecific hybrids. When two species do not normally hybridize in nature but F_1 hybrids can be produced in the laboratory, genetic differences will express themselves as incompatibilities; that is, epistatic interactions between the alleles that can result in morphological/physiological abnormalities and a loss of fitness. What our present data clearly suggest is that interspecific hybrids are as able as their parents at buffering developmental noise (i.e. genuine FA did not appreciably increase), notwithstanding the fact that hybrid's proper bilateral development can be harshly compromised.

Canalization was originally intended to describe the ability to develop a target phenotype despite genetic or environmental perturbations (Waddington 1942, 1952). Viewed from this perspective, the range of morphological anomalies observed in *D. madeirensis–D. subobscura* hybrid females (including the substantial wing size asymmetry that would obviously impair aerodynamic properties) clearly indicates that buffering mechanisms that stabilize development against genetic perturbations (i.e., disruption of coadaptation) appear to be largely independent of those mechanisms that buffer against developmental noise. Certainly the genetic perturbations in the interspecific hybrids could be so strong that the observed abnormalities are outside the normal region of canalization. Waddington (1942) noted that buffering occasionally breaks down, and the phenotypic expression of cryptic genetic variation after inhibition of Hsp90 activity is a recent example (Rutherford and Lindquist 1998; Queitsch et al. 2002). Furthermore, inhibiting Hsp90 activity does not increase FA (Milton et al. 2003), in qualitative agreement with our observations. A small relationship between genetic canalization and developmental stability was also apparent when comparing the VCV matrices for the genetic components of the interindividual variation (e.g., the sire component) to those for FA (Table 4). In summary, our results add to a growing body of recent empirical evidence suggesting that the underlying processes that control (genetic) canalization and developmental stability do not share a common mechanism (Debat et al. 2000; Milton et al. 2003; Pélabon et al. 2004; Dworkin 2005a,b; Santos et al. 2005; but see Willmore et al. 2005).

To conclude, we argue here against the conventional tenet that developmental instability is expected to increase when coadapted gene complexes are broken down once interspecific hybrids are formed. A quantitative genetics framework is needed to appropriately address the relationship between hybridization, canalization, and the phenotypic outcome in bilateral traits once purely stochastic variation has been filtered by the developmental system (FA; Leamy and Klingenberg 2005). *Drosophila* hybrids also provide an invaluable material to approach those issues from a developmental genetics and functional genomics perspective.

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This paper is dedicated to M. Monclús, who first described *Drosophila madeirensis* and *D. guanche* after her trips to collect drosophilids in the Macaronesian Islands (Azores, Canaries, Madeira) during 1969–1970. We thank I. Fragata for technical assistance and L. Serra for encouraging and useful remarks on the work. The constructive criticisms from associate editor and two anonymous reviewers on earlier drafts were of invaluable help to substantially improve the paper. We are particularly grateful to the associate editor for illuminating discussions as to appropriately correct for FA bias in our half-sib hybrid design. CR received a grant from Fundação para a Ciencia e Tecnologia (PRAXIS XXI/BD/21479/99). MS is partially supported by Fundación Ramón Areces (Spain). This work was accomplished within the Acción Integrada Hispano-Portuguesa HP2003-0099 and the Acções Integradas Luso-Espanholas E-36/04.

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Chapter 5.

Comparing adaptive potential in two *Drosophila* species

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Do Species Converge during Adaptation? A Case Study in *Drosophila*

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ABSTRACT

Adaptation to novel environments is a crucial theme in evolutionary biology, particularly because *ex situ* conservation forces populations to adapt to captivity. Here we analyze the evolution of life-history traits in two closely related species, *Drosophila subobscura* Collin and *Drosophila madeirensis* Monclús, during adaptation to the laboratory. *Drosophila madeirensis*, an endemic species from Madeira, is here shown to have less ability to adapt to the laboratory. Early fecundity was the only trait where this species showed a significant improvement with time. By comparison, *D. subobscura* improved in most traits, and its early fecundity increased faster than that of *D. madeirensis*. Our findings suggest that different species, even closely related ones, may adapt at different rates to the same environment.

Introduction

Many studies of experimental evolution in *Drosophila* have focused on adaptive divergence in response to a diversity of selection regimes starting from common ancestral populations (for reviews, see Rose et al. 1996; Prasad and Joshi 2003; Chippindale 2006). Adaptive convergence has also been studied using experimental evolution; the typical experimental strategy is to follow the evolutionary trajectories of initially divergent populations undergoing selection in a common environment (e.g., Matos et al. 2000, 2002, 2004; Teotónio and Rose 2000; Teotónio et al. 2002).

In general, studies of convergence in single species suggest that the functional characters of initially evolutionarily differentiated populations usually converge when they are maintained in a common environment for many generations. However, whether or not convergence occurs depends on the trait analyzed and on the previous history of selection (Teotónio et al. 2002). The rate of convergence varies as a function of the initial differentiation between the populations (e.g., Teotónio and Rose 2000; Matos et al. 2002; Simões et al. 2007). It is possible that different genetic backgrounds might eventually lead to divergence between populations adapting to similar environments (Cohan 1984*a*, 1984*b*), particularly in different species (Cohan and Hoffmann 1989). What happens when populations of different species come together in a similar environment? Will they converge, adapting in the same manner to similar conditions, or will they evolve toward different adaptive peaks, as a consequence of different genetic backgrounds?

Understanding the evolutionary changes involved in adaptation to controlled environments is particularly important for conservation efforts, especially when captive breeding is involved. Captive breeding is essential for the conservation of many species (Frankham et al. 1986; Ralls and Ballou 1986; Soulé et al. 1986; Tudge 1995; Frankham 2002). Captivity over multiple generations involves evolutionary changes, and some of these changes can be detrimental to reintroduction (Woodworth et al. 2002). For example, the genetic changes that maximize fitness in captivity could be deleterious in the native environments (Frankham et al. 1986; Frankham 2002). Inbreeding, accumulation of deleterious mutations, and loss of genetic variation are other types of genetic problems that can arise among captive breeding populations (Frankham 2002; reviewed in Frankham 2005*a*). A common approach to studying the problems associated with adaptation to captivity has been to study model organisms like *Drosophila* species rather than endangered species themselves (e.g., Frankham 1995; Woodworth et al. 2002; Gilligan and Frankham 2003).

Here we study the laboratory evolution of populations of two *Drosophila* species derived from collections in the wild. *Drosophila madeirensis* Monclús is an endemic species from Madeira Island. *Drosophila subobscura* Collin, its close relative, is a species with a much wider distribution. Both species coexist in sympatry on Madeira Island, despite being morphologically very similar (Monclús 1984). The estimated time of divergence for this species pair is 0.6–1 million years (Ramos-Onsins et al. 1998). Their reproductive isolation is incomplete, as it is possible to obtain viable and fertile hybrids, especially when *D. madeirensis* is the mother species (Khadem and Krimbas 1991, 1993; Papaceit et al. 1991; Rego et al. 2006).

Drosophila madeirensis is associated with a particular type of habitat, the Laurisilva forest, considered a relic of the subtropical

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Table 1: Differences between species tested by nested ANOVA (with replicates nested within species)

Generation	a1r	F1–7	F8–12	RM	RF
7	.039[a]	4.15[a]	1.16[a]	8.401*	3.392[a]
14	1.685[a]	13.24*	32.971**		
23	15.888*	68.47**	49.248**		
43	15.069*	254.793***	2246.344***	17.902*	17.668*

Note. The $F_{1,4}$ values and significance level are presented. a1r = age of first reproduction; F1–7 = early fecundity; F8–12 = peak fecundity; RM = male starvation resistance; RF = female starvation resistance.

[a] Not significant.

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

forests that in the Tertiary era covered the Circum-Mediterranean area. Madeiran Laurisilva has recently been inscribed in the World Heritage List (IUCN 1999) due to its outstanding natural value as the largest surviving area of this type of ecosystem. However, its distribution is decreasing drastically as a result of human activity, a fact that is endangering many animal and plant species, particularly species that are endemic, like *D. madeirensis*.

By bringing populations of both of these *Drosophila* species into a new common environment with controlled laboratory conditions, we seek to answer the following questions: How differentiated are these species when reared in a common environment, and is this differentiation maintained during laboratory evolution, or do *D. subobscura* and *D. madeirensis* converge or diverge during adaptation to captivity?

Material and Methods

Populations

In April 2001, laboratory populations of *Drosophila madeirensis* and *Drosophila subobscura* were established using as founders wild individuals collected in a patch of Laurisilva forest near Ribeiro Frio, Madeira. Since their foundation, these populations were maintained in controlled conditions of 18°C with a photoperiod of 12L : 12D. The maintenance regime involved discrete generations of 30 d, with controlled adult and larval densities (50 adults/vial, 70–90 eggs/vial). The average number of individuals per generation was 1,000, never dropping below 400 for each population (for more maintenance details see Matos et al. 2000, 2002). When the populations were in their third generation of laboratory culture, they were split into three replicate populations for each species (from here on called m1, m2, and m3 for *D. madeirensis* and s1, s2, and s3 for *D. subobscura*).

Assays

Fecundity assays were carried out in the following manner: in each of the generations, analyzed mating pairs were formed using virgin individuals from each replicate population, and the sample sizes varied from 12 to 24 pairs. Pairing was done with CO_2 anesthesia during the first 6 h after adult eclosion to guarantee that the individuals were virgin at the time of sample formation. The daily fecundity of each mating pair was recorded over 12 d. In some generations, starvation resistance was also analyzed. To do this, after the fecundity assay, the mating pairs were transferred to vials containing plain agar, and the time of death was estimated using observations every 6 h. Fecundity assays were carried out on generations 7, 14, 23, and 43. Generations 7 and 43 also included starvation resistance assays. At generation 43, the Madeiran populations of *D. madeirensis* and *D. subobscura* were assayed in synchrony with three sets of threefold replicated continental populations of *D. subobscura*. One set of populations, derived from a foundation in Sintra in 2001, is called TW. Another set was founded simultaneously from a collection in Arrábida and is called AR. The third set of populations also derived from Sintra from an earlier foundation in 1990 and is called NB. By the time this assay was done, the TW and AR continental populations had undergone 40 generations in the laboratory while the NB populations were in their 176th generation of laboratory culture (see details in Matos et al. 2004).

The traits analyzed were age of first reproduction (a1r; number of days before the first egg laying), early fecundity (F1–7; number of eggs laid in the first 7 d), peak fecundity (F8–12; number of eggs laid in the last 5 d of the study), and starvation resistance (RF and RM, for females and males, respectively; number of hours an individual resisted without food). Daily fecundity, the number of eggs laid by the females in each day, was also analyzed.

Statistical Analysis

For each assayed generation, a two-way nested ANOVA was performed to test whether the differences between species were significant, with replicate populations (random effect) nested within species. This analysis was done separately for each trait assayed.

Daily Fecundities. For each assay, daily fecundities over multiple days were analyzed by plotting the mean daily fecundity values against the age of the females and estimating the best regression model for each replicate population of both species. Two types of regression models were estimated: linear and second-degree polynomial. The best-fit model was chosen according to the Akaike Information Criterion (AIC; i.e., Bieri and Kawecki 2003). When a linear model was the best-fit model for all replicate populations of each species, *t*-tests were applied to test whether the trend was significant. These *t*-tests were done on the average slopes for each species using the variation of slopes between replicates as the sample variation. The same criterion was applied when comparing the two species.

The mean daily fecundities of both species were also compared with *t*-tests performed for each day. Significance levels were adjusted with a sequential Bonferroni technique using available software (Rice 1989).

Starvation Resistance. At generations 7 and 43, *t*-tests were used to compare starvation resistance between species, using the heterogeneity between replicates as the source of error. We also did a two-way ANOVA with species (fixed) and assayed generation (random) as factors, testing for overall differences between species, differences between assayed generations, and changes between species across the generations assayed.

Comparison with Continental Populations. At generation 43, Madeiran populations were compared with continental populations of *D. subobscura* during a synchronous assay. *Drosophila madeirensis* was compared with each replicated set of *D. subobscura* (Madeira, Sintra [TW and NB], and Arrábida [AR]) populations using *t*-tests on the mean values for all traits assayed. The same procedure was applied when comparing *D. subobscura* (Madeira) with continental populations. *P* values were adjusted by a sequential Bonferroni method (Rice 1989). Specifically, for comparisons between *D. madeirensis* and *D. subobscura* from each foundation, *P* values were adjusted considering the use of four tests, and for comparisons between *D. subobscura* from Madeira and the continent, *P* values were adjusted considering the use of three tests. In all cases, the estimated error was based on the heterogeneity between replicates within each set of populations. *Drosophila madeirensis* was also compared with all *D. subobscura* populations founded at a similar time (*D. subobscura* [Madeira], TW, and AR), using the average of the three mean values estimated for each trait and set of populations. In these tests, the variance for *D. subobscura* was estimated as the heterogeneity between different foundations (differences between the means of the three sets of populations independently founded).

Evolutionary Trajectories. Type I least squares linear regressions were carried out to analyze the evolutionary trajectories of each species, with the mean values of each trait as the dependent variable and generation as the independent variable (Sokal and Rohlf 1995). Regression models were obtained independently for each of the three replicate populations of each species. To evaluate whether there was a consistent, directional, linear change over evolutionary time, a *t*-test was performed on the

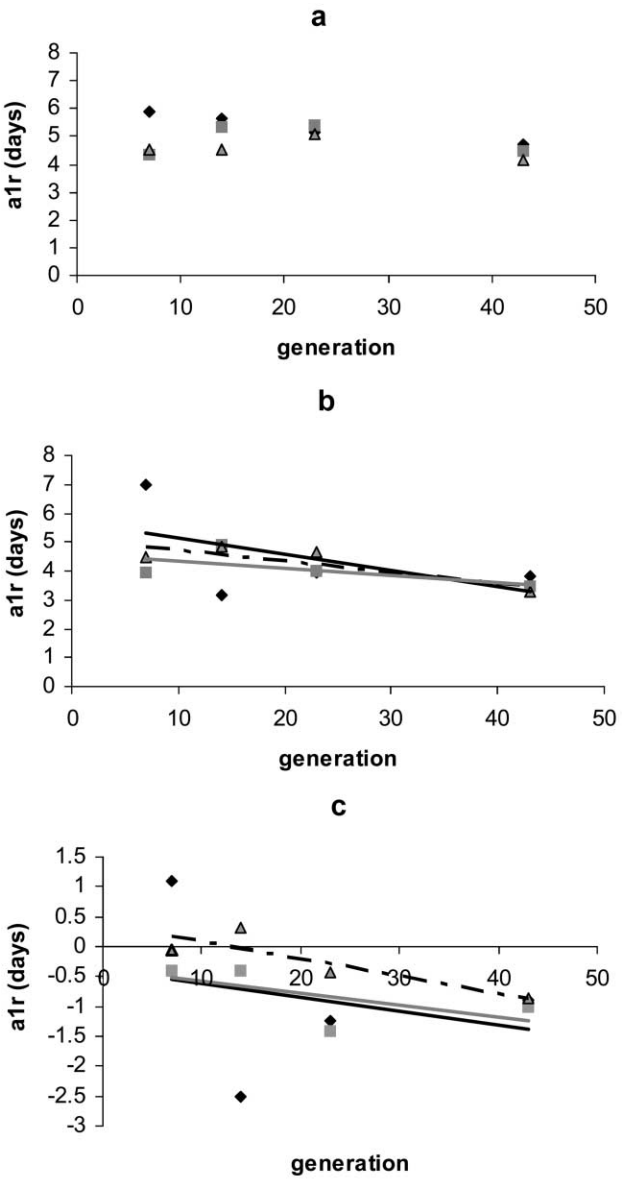

Figure 1. Evolutionary trajectories for age of first reproduction. Plots of means of age of first reproduction (a1r) as a function of generation number for *Drosophila madeirensis* (*a*), for *Drosophila subobscura* (*b*), and for differences between them (*D. subobscura* − *D. madeirensis*; *c*). Data points show the mean values of replicate populations of each species. Significant linear trends (presented) were obtained for *D. subobscura* ($P < 0.05$; *b*) and for the differences between the two species ($P < 0.01$; *c*). *Black line*, *diamonds*, replicate 1; *gray line*, *squares*, replicate 2; *broken black line*, *triangles*, replicate 3.

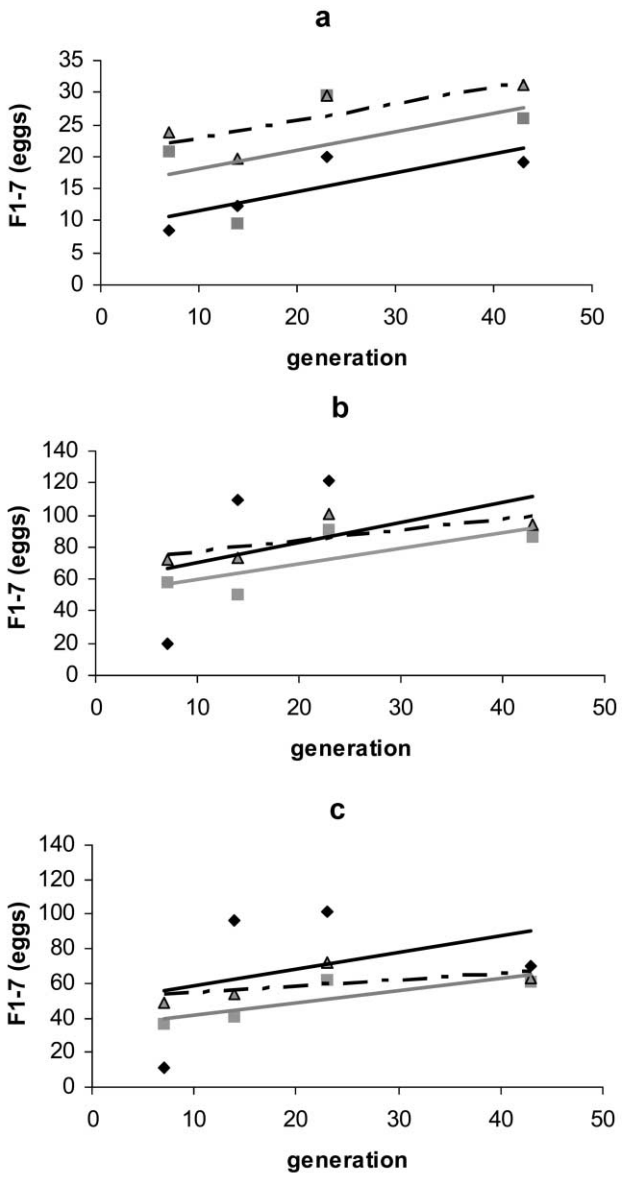

Figure 2. Evolutionary trajectories for early fecundity. Plots of means of early fecundity (F1–7) as a function of generation number for *Drosophila madeirensis* (*a*), for *Drosophila subobscura* (*b*), and for differences between them (*D. subobscura* − *D. madeirensis*; *c*). Data points show the mean values of replicate populations of each species. Significant linear trends (presented) were obtained for *D. madeirensis* ($P < 0.001$; *a*), for *D. subobscura* ($P < 0.05$; *b*), and for the differences between the two species ($P < 0.05$; *c*). *Black line*, *diamonds*, replicate 1; *gray line*, *squares*, replicate 2; *broken black line*, *triangles*, replicate 3.

average slope among the populations of each species, using the variation of slopes as sample variation.

The same procedure was applied to the differences between pairs of replicates from each species. Least squares linear regressions were done using as data points the differences between same numbered replicate populations from each species (e.g., s1-m1) to test for temporal variation in the differences between them. Finally, an ANCOVA was used to test for homogeneity of slopes between the two species, with species as a factor with two categories (*D. madeirensis* and *D. subobscura*) and generation as the covariate.

Temporal change in the patterns of daily fecundity was also analyzed. The slopes of the linear regressions of daily fecundity obtained for each replicate population were plotted against generation number. A linear regression was estimated to see whether there was a trend in the temporal change of this trait, the significance of this trend being estimated by a *t*-test. A similar procedure was used to test for temporal changes in the differences between slopes of the two species. All statistical analysis was done using STATISTICA and EXCEL.

Results

Fecundity Traits

The results of the nested ANOVA indicate that in general the species differ significantly in all fecundity-related traits in all generations. In the cases where the differences were not significant, this was mostly due to a higher heterogeneity between replicates, which was common in the earlier generations, particularly generation 7 (Table 1). The same may explain the lack of significant differences for age of first reproduction at generation 14. In all instances, *Drosophila subobscura* had higher fecundity and quicker maturation time relative to *Drosophila madeirensis* (Figs. 1–3).

Daily Fecundities

Figure 4 presents a plot of daily fecundities against age for each species and generation. The change of daily fecundity with age differs between the species. *Drosophila madeirensis* presents the same pattern in all assayed generations, with an initial stage without laying eggs followed by a steady increase in fecundity. In all generations the best-fit model for daily fecundity is a linear regression for this species (Fig. 4). *Drosophila subobscura* has a similar pattern in generation 7, but in subsequent generations, it presents a pattern that is best fit by a second-degree polynomial regression (after application of AIC). This pattern includes two phases: an initial maturation period including the beginning of egg laying followed by an increase in fecundity, reaching an apparent peak around days 8–10 of the assay, after which there is a drop in fecundity (Fig. 4).

All the models obtained for each replicate population were highly significant. The *t*-tests performed on the average slope for *D. madeirensis* in all assayed generations also gave significance (generation 7: average slope = 2.184, $t = 20.798$, $P < 0.01$; generation 14: average slope = 0.609, $t = 10.263$, $P < 0.01$; generation 23: average slope = 1.418, $t = 11.766$, $P <$

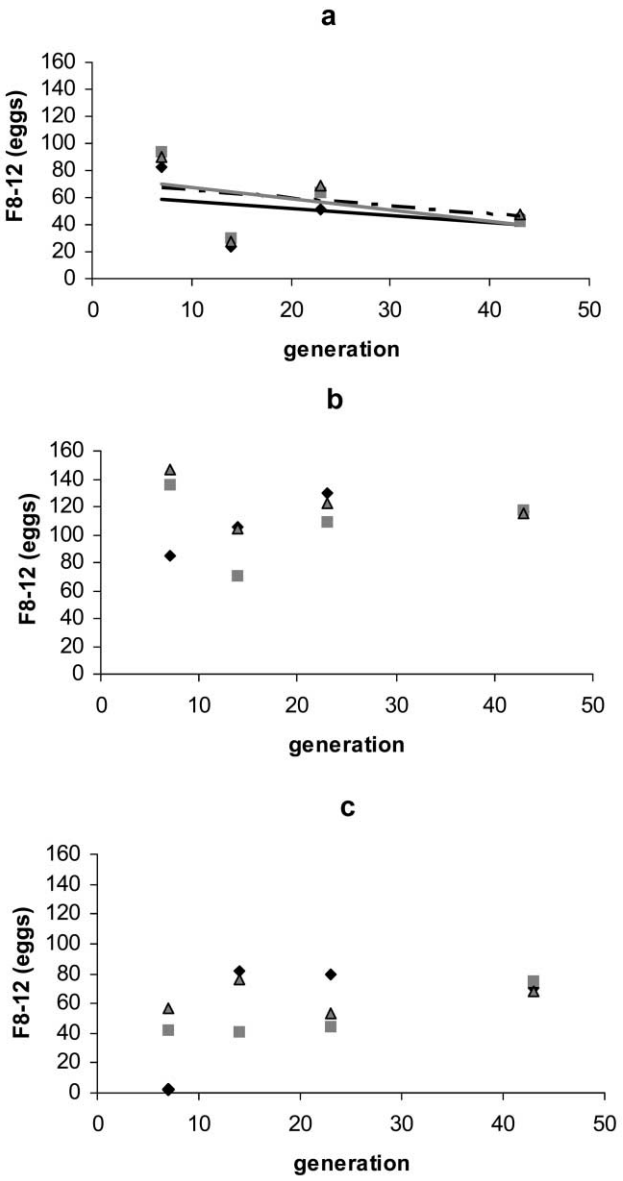

Figure 3. Evolutionary trajectories for peak fecundity. Plots of means of peak fecundity (F8–12) as a function of generation number for *Drosophila madeirensis* (*a*), for *Drosophila subobscura* (*b*), and for differences between them (*D. subobscura* − *D. madeirensis*; *c*). Data points show the mean values of replicate populations of each species. A significant linear trend (presented) was obtained for *D. madeirensis* ($P < 0.05$; *a*). *Black line*, *diamonds*, replicate 1; *gray line*, *squares*, replicate 2; *broken black line*, *triangles*, replicate 3.

0.01; generation 43: average slope = 0.952, $t = 22.504$, $P < 0.01$). The same test performed with the data from *D. subobscura* for generation 7 presents similar results (average slope = 2.727, $t = 4.927$, $P < 0.05$). There were no significant differences between the slopes obtained for both species in generation 7 ($t = 0.965$, $P > 0.5$).

Also performed were *t*-tests comparing the average fecundity of both species day by day, the *P* values being adjusted using a sequential Bonferroni correction (Rice 1989). The results indicate that on generation 7, both species had similar fecundity values during the assayed period, the first 12 d of adulthood. However, in the following generations, *D. subobscura* in general presented higher fecundities throughout the assay. At generation 14, *D. subobscura* presented higher fecundities between days 5 and 9, while in the remaining days of the assay, no significant differences were found. Finally, at generations 23 and 43, *D. subobscura* presented significantly bigger fecundities from day 5 on ($P < 0.05$). Overall, these results suggest a tendency for divergence between the two species during adaptation to the laboratory, with *D. subobscura* exhibiting a more rapid increase in the new environment.

Starvation Resistance

Figure 5 presents the average values of male and female starvation resistance for both species during the two assayed generations. *Drosophila madeirensis* had higher starvation resistance in both assays and for both sexes. The nested ANOVA results indicate that *D. madeirensis* flies were significantly more resistant in both generations, the exception being female starvation resistance in generation 7. A bifactorial ANOVA comparing the two species and the two assayed generations indicates that both female and male starvation resistance differed significantly between species (RF: $F_{1,8} = 17.413$, $P < 0.001$; RM: $F_{1,8} = 34.985$, $P < 0.0001$) as well as between assays (RF: $F_{1,8} = 51.069$, $P < 0.0001$; RM: $F_{1,8} = 9.878$, $P < 0.05$). Over both species, starvation resistance achieved higher values in generation 7. The interaction term was not significant, which seems to indicate that both species did not diverge significantly in terms of starvation resistance during laboratory adaptation.

Comparisons with Continental Populations

The assay carried out on generation 43 was done in synchrony with an assay performed on other *D. subobscura* populations derived from the continent: the TW, AR, and NB populations. The results of unpaired *t*-tests on the differences of averages between the different sets of populations (using the heterogeneity among replicates as source of error) are given in Table 2. In general, *D. madeirensis* differs from continental populations of *D. subobscura* for all fecundity-related traits, having a poorer performance in that they started to lay eggs later and laid fewer eggs. *Drosophila madeirensis* males are in general more starvation resistant. On the other hand, no significant differences were found for female starvation resistance between *D. madeirensis* and continental *D. subobscura* populations. The only trait where continental and Madeiran populations of *D. subobscura* differed was peak fecundity, continental populations laying more eggs independently of geographic origin and num-

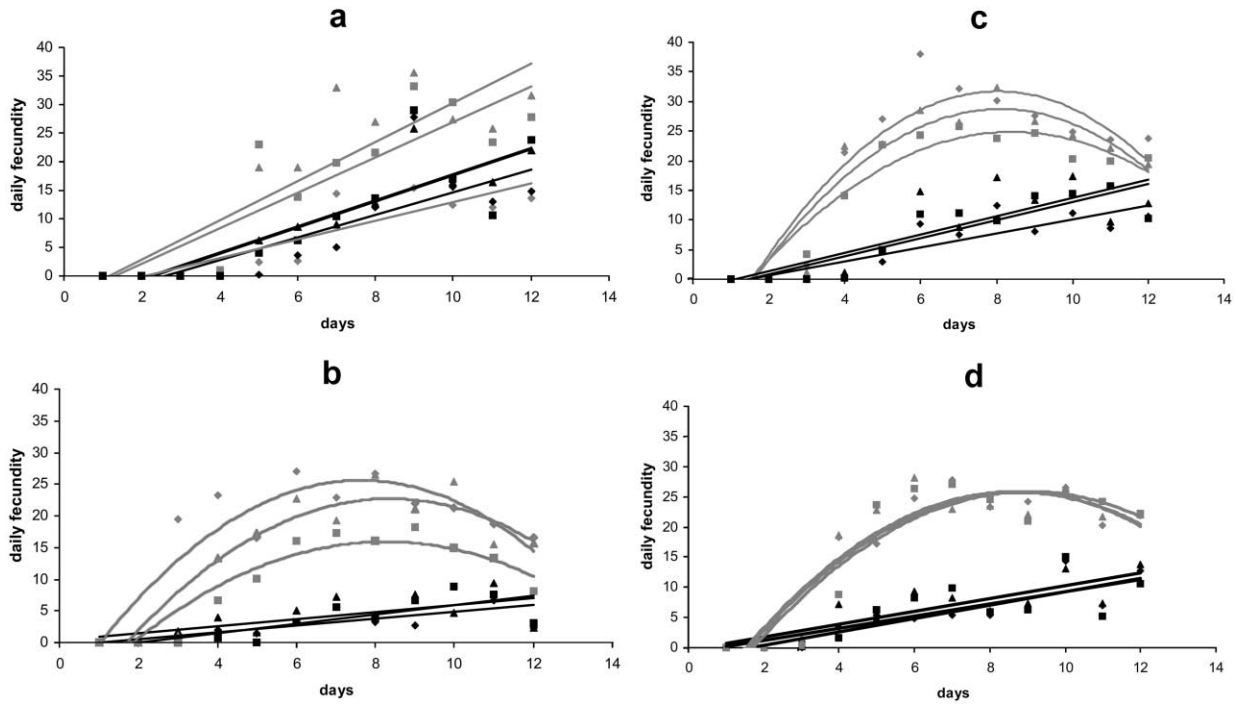

Figure 4. Daily fecundities for each of the generations assayed. Mean daily fecundities of *Drosophila madeirensis* and *Drosophila subobscura* replicate populations in generations 7 (*a*), 14 (*b*), 23 (*c*), and 43 (*d*). The best regression models for each replicate population are also shown. *Diamonds*, s1, m1; *squares*, s2, m2; *triangles*, s3, m3; *gray*, *D. subobscura*; *black*, *D. madeirensis*.

ber of generations in the laboratory (Table 2). Significant differences in all traits are shown in *t*-tests on the overall differences between *D. madeirensis* and *D. subobscura* (using as source of error of the latter different foundations), both continental (specifically TW and AR) and Madeiran. In these comparisons, *D. subobscura* presented quicker maturation, higher fecundity, and lower starvation resistance, paralleling the differences between *D. subobscura* from Madeira and *D. madeirensis* (Table 2). This conclusion is still valid applying a sequential Bonferroni adjustment for five tests.

Evolutionary Trajectories

Figures 1–3 show the temporal changes of age of first reproduction, early fecundity, and peak fecundity for both species as well as differences between species. To check for significance of linear evolutionary trajectories, *t*-tests were performed on the average slope for each species and trait (Table 3). Age of first reproduction (a1r) significantly evolved in *D. subobscura*, with females laying eggs progressively earlier. The same tendency occurred in *D. madeirensis*, though it was not statistically significant (Fig. 1). Early fecundity (F1–7) increased significantly in both species (Fig. 2). On the other hand, peak fecundity (F8–12) declined significantly in *D. madeirensis* but not in *D. subobscura* (Fig. 3).

The analysis of the effect of laboratory evolution on the differentiation of the two species indicates that there is a significant divergence for age of first reproduction and early fecundity, with *D. subobscura* increasing its performance at a higher rate relative to *D. madeirensis* (Table 3; Figs. 1, 2). The same tendency occurs for peak fecundity, though it is not significant (Table 3; Fig. 3). Nevertheless, the average slopes of these two characters did not differ significantly when tested for parallelism by ANCOVA (Table 4). This lack of significance is probably due to a lack of statistical power because the absolute values of replicates were used in this statistical comparison rather than analysis of differences between pairs of populations assayed synchronously.

Evolutionary change was also analyzed using daily fecundity data. The slopes of daily fecundity obtained for *D. madeirensis* replicate populations in each generation were plotted against generation number, and a linear regression was applied. A *t*-test performed on the average slope indicates that the slopes of daily fecundity decreased with time ($t = 6.05$, $df = 2$, $P = 0.026$). This suggests that the increase in daily fecundity with age decreased during the adaptation to the new environment. This may be due to a drop in fecundity at later ages relative to its level during the first few days of egg laying (see above).

To analyze the temporal change in daily fecundity for *D. subobscura*, a similar approach was used. The slopes of linear

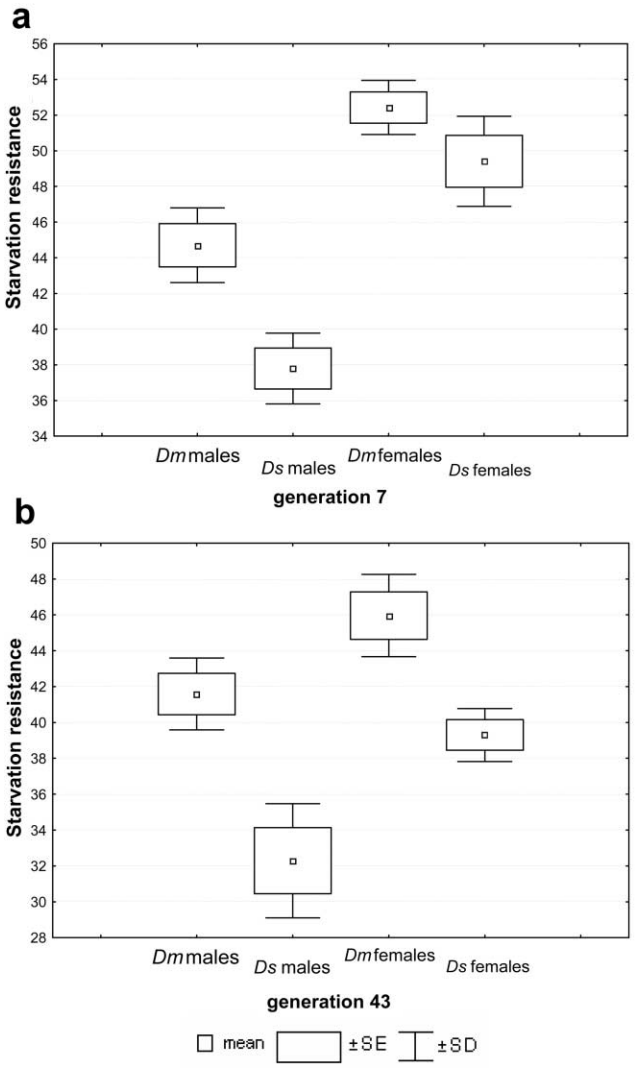

Figure 5. Starvation resistance over different generations. Box plot of female and male starvation resistance values of *Drosophila madeirensis* and *Drosophila subobscura* in generations 7 (*a*) and 43 (*b*). The mean values of each replicate population were used as individual data points.

regressions obtained for daily fecundity for each replicate population were plotted against generation number, and another linear regression was applied. The *t*-test results on the average slope indicate that the pattern of increase in daily fecundity did not change during laboratory adaptation ($t = 1.01$, df $= 2$, $P = 0.42$). The fact that this species does not show a detectable increase in this character during laboratory evolution may be an artifact arising from a lack of applicability of linear regression models for daily fecundity in this species.

We have nevertheless tested whether there was a temporal change in the differences between species relative to a linear pattern of increase in daily fecundity. In each generation assayed, we estimated the differences in the slopes of the linear model adjusted for daily fecundity data for both species (pairing same-numbered replicate populations, e.g., s1, m1). A linear regression was applied to these data as a function of generation. The results indicate that overall there were no evolutionary changes in the differentiation of these species with respect to the dependence of daily fecundity on age (data analysis not shown).

Discussion

Comparing Initial Laboratory Adaptation in Two Species

Drosophila subobscura and *Drosophila madeirensis* differ greatly in terms of fecundity; *D. subobscura* has quicker maturation and lays more eggs during the assayed periods. The differences between the two species for daily fecundity could be due to their maturation time. As *D. madeirensis* females start laying eggs later, it is possible that including more days in the assay would give *D. madeirensis* the opportunity to express a similar fecundity pattern to the one observed in *D. subobscura*, albeit with a time delay. For some of the generations analyzed in this study, daily fecundity data for two additional days were available, but these later daily fecundities also showed superiority of *D. subobscura*.

Why are these species so persistently different with respect to fecundity in the laboratory environment? The differences that we have observed could mean that these two species explore different resources in their natural environments. Maintaining both species in the same conditions might favor one species relative to the other. In the case of our study, *D. subobscura* could have been unintentionally favored because the maintenance conditions that we adopted were based on our prior experience with this species (Matos et al. 2000, 2002). Furthermore, the fact that *D. madeirensis* is an endemic species, apparently specialized to the Laurisilva forest, while *D. subobscura* is a widespread species found in a large variety of habitats, suggests that we are dealing with two species that differ in their ability to exploit novel resources (Parsons and Stanley 1981; Parsons 1982). Nevertheless, our field experience indicates that both species can be collected simultaneously in the same baited trap (C. Rego, unpublished data). This suggests that both species share feeding and/or breeding preferences.

Drosophila madeirensis flies in general have higher starvation resistance. This may be partly due to their bigger size (Rego et al. 2006). In fact, such an association was found in a comparison of several *Drosophila* species by Sharmila Bharathi et al. (2003). Nevertheless, more studies are needed to test for this pattern.

An important issue to bear in mind is that the populations studied here are undergoing adaptation to a novel environment. It is possible that fecundity may vary between our two species as a function of how much they are preadapted to recognize our culture medium as adequate for egg laying, while starvation resistance basically differs due to different sizes (Sharmila Bharathi et al. 2003). This does not preclude the possibility that

Table 2: *t*-tests on the differences between mean values of the several life-history traits assayed and their respective significance level

	a1r	F1–7	F8–12	RM	RF
Drosophila madeirensis vs. *Drosophila subobscura*:					
D. madeirensis vs. *D. subobscura* (Madeira)	3.885*	16.164***	47.810***	4.276*	4.223[a]
D. madeirensis vs. NB	4.246*	−13.818***	−35.652***	7.371**	2.647[b]
D. madeirensis vs. TW	7.321**	−9.316**	−23.978***	2.628[a]	2.637[b]
D. madeirensis vs. AR	3.797*	−6.287**	−11.923 ***	6.589**	2.122[b]
D. madeirensis vs. *D. subobscura* (Madeira)+ TW + AR	4.776**	−11.809***	−6.05**	3.952*	4.296*
D. subobscura (Madeira) vs. *D. subobscura* (Continent):					
D. subobscura (Madeira) vs. NB	1.807[b]	−1.236[b]	−10.997***	.0486[b]	−.542[b]
D. subobscura (Madeira) vs. TW	2.677[b]	−1.236[b]	−15.888***	−2.148[b]	−.547[b]
D. subobscura (Madeira) vs. AR	.294[b]	.656[b]	−4.530*	−.559[b]	−.508[b]

Note. *t*-tests compare *Drosophila madeirensis* with *Drosophila subobscura* from different geographical origins, *D. madeirensis* with the average for *D. subobscura* across synchronous foundations, and *D. subobscura* from Madeira with Continental populations. The *P* values were corrected using a sequential Bonferroni method. See note to table 1. NB, TW = *D. subobscura* from Sintra (two independent foundations; see details in the text); AR = *D. subobscura* from Arrábida.

[a] $0.05 < P < 0.06$.

[b] Not significant.

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

genetic trade-offs may also play a role. On the other hand, the two species may also differ in their capacity to assimilate the nutrients that we have provided. Such a difference might affect both fecundity and resistance in a similar way, giving rise to a positive covariance between traits (Service and Rose 1985; Matos et al. 2000). Nevertheless, all these inferences deserve a note of caution, given that comparisons between two species do not allow direct inference of the genetic architecture within each population, given that they have different genetic backgrounds (Leroi et al. 1994).

Evolutionary Trajectories during Laboratory Adaptation

Overall, our results indicate a general tendency for improvement in characters related to fecundity, particularly early reproduction. Both species showed a tendency to improve in both early fecundity and age of first reproduction during adaptation to the new environment, the rate being higher in *D. subobscura*. This is in accordance with other studies of laboratory adaptation that indicate an increase in early fecundity; most of these studies focus on a single species (*D. melanogaster*, Sgrò and Partridge 2000; *D. subobscura*, Matos et al. 2000, 2002; but see Hercus and Hoffmann 1999 for a study of *Drosophila birchii* and *Drosophila serrata* and their hybrids). On the other hand, peak fecundity did not show any consistent evolutionary change in *D. subobscura*, while it actually declined in *D. madeirensis*. This is an unexpected result, given our other studies in *D. subobscura* (e.g., Matos et al. 2002). It might be explicable in terms of either differences in genetic background or founder effects, two causes that a subsequent study might unravel. Of course, these results need to be interpreted with caution given that transient assay effects may have also contributed to the patterns observed in these analyses of absolute values.

Again, the difference in the rate of adaptation of early fecundity between the two species could be due to the fact that we are dealing with two species with different ecological requirements, *D. subobscura* being a widespread, generalist species and *D. madeirensis* being an endemic species, specialized on Laurisilva forest. Widespread species are expected to be resource generalists and as such to have a higher ability to adapt to new conditions during domestication (Parsons 1982). Adaptive evolutionary potential is in general dependent on quantitative genetic variation (Frankham 1995, 2005*b*). This is in turn expected to be lower in populations with a more restricted geographic distribution, including fragmented habitats (Lienert et al. 2002). *Drosophila madeirensis*, being more specialized and appearing only on Laurisilva forest patches, may indeed give it less potential to adapt to novel environments because of lower genetic variability. The fact that it adapts more slowly than *D. subobscura* in our laboratory corroborates this hypothesis.

The results suggest that starvation resistance decreases during laboratory adaptation in both species. Our previous work and that of others has revealed some inconsistencies in the evolution of starvation resistance during domestication (e.g., Hoffmann et al. 2001; Matos et al. 2002, 2004; Griffiths et al. 2005; Simões et al. 2007). Though in this study we obtained a suggestion of a parallel decline in starvation resistance in the two species, this should be interpreted very cautiously because we only compared starvation resistance at two points in the evolutionary process. Therefore, there is no firm generalization to be made

about the comparative laboratory evolution of starvation resistance. There was no sign of either progressive divergence or progressive convergence between species during the laboratory evolution of starvation resistance in a common environment, unlike previous results with populations of a single species (e.g., Teotónio and Rose 2000).

On balance, the experimental evolution of early fecundity clearly indicates adaptation to the new environment in both species. *Drosophila subobscura* also shows signs of improvement for age of first reproduction. On the other hand, the observed patterns for age of first reproduction, peak fecundity, and daily fecundity for *D. madeirensis* suggest a possible failure to adapt, a failure that might eventually lead to cumulative divergence between the two species (see below).

Table 4: ANCOVA results comparing average values for each species and trait in each generation using generation as a covariate

	a1r	F1–7	F8–12
Generation	6.041*	4.710*	.598[a]
Species	4.478*	59.277***	37.787***
Generation × species	1.072[a]	1.457[a]	1.300[a]

Note. *F* values and significance levels are given. a1r = age of first reproduction; F1–7 = early fecundity, F8–12 = peak fecundity. The *F* values of tests on the homogeneity of slopes (generation × species), comparing both species and their significance levels, are also given.

[a] Not significant.

* $P < 0.05$.

*** $P < 0.001$.

Do Species Converge under Similar Conditions?

Our data indicate that *D. subobscura* and *D. madeirensis* were different with respect to several life-history traits from the moment they were brought into the laboratory. Subsequent laboratory evolution produced no apparent convergence. On the contrary, we found signs of evolutionary divergence between them, though this differentiation varies from trait to trait. It is unlikely that the slower rate of improvement of *D. madeirensis* was due to higher inbreeding levels during laboratory culture compared with *D. subobscura*, given that they were maintained at similar population sizes.

Table 3: Slopes of the linear evolutionary trajectories for each trait and replicate population

	a1r	F1–7	F8–12
m1	−.034	.293	−.510
m2	−.005	.283	−.860
m3	−.010	.263	−.608
Average	−.016[a]	.280***	−.659*
s1	−.057	1.259	.781
s2	−.025	.979	.103
s3	−.038	.654	−.511
Average	−.040*	.964*	.124[a]
s1 − m1	−.028	.966	1.291
s2 − m2	−.019	.696	.962
s3 − m3	−.023	.390	.096
Average	−.024**	.684*	.783[a]

Note. The average values for *Drosophila madeirensis* (m1, m2, m3), for *Drosophila subobscura* (s1, s2, s3), and for the differences between both species as well as the significance levels (*t*-tests) are also given. a1r = age of first reproduction; F1–7 = early fecundity; F8–12 = peak fecundity.

[a] Not significant.

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

Our data suggest that the evolution in a novel, common environment increases differences between species that are already expressed at foundation. This may be a result of different evolutionary dynamics resulting from different genetic backgrounds, which is particularly expected when dealing with different species (Cohan and Hoffmann 1989). However, variation within species can confound interspecific comparisons, especially in species with wide distributions (Hoffmann and Harshman 1999). We have obtained evidence of effects of foundation in previous studies of the evolutionary dynamics of *D. subobscura* populations (Matos et al. 2002; Simões et al. 2007). It would thus be important to test for repeatability of the results obtained in this study using several independent foundations from both *D. subobscura* and *D. madeirensis*.

Implications for Captive Breeding

There is a lack of previous empirical studies estimating how much species differ in their evolutionary rates during adaptation to captivity, though some studies are tangentially relevant (e.g., Deckert-Cruz et al. 2004). Our data suggest that generalization from one species to another, even to closely related species, can be misleading. Adaptation to captivity may occur generally, but its rate depends on the genetic background of each species. This could be particularly relevant for conservation efforts, because some species may fail to thrive in captivity due to an inability to adapt to such novel conditions.

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Chapter 6.

DISCUSSION

Discussion is an exchange of knowledge; Argument an exchange of ignorance.

Robert Quillen

A conclusion is the place where you got tired of thinking. Harold Fricklestein

Speciation and species differentiation are some of the more interesting fields in Evolutionary Biology, and have been very productive judging by the amount of books and research papers published in this area in recent years (reviewed in Coyne and Orr 2004).

With the work here presented I intended to obtain answers to several questions regarding the differentiation between *Drosophila madeirensis* and *Drosophila subosbcura*, two closely related species with incomplete reproductive isolation. Considering the controversy surrounding the relative relevance of additive and non-additive genetic effects in population differentiation, I started by asking if these two species are differentiated in terms of life-history traits, what is the importance of additive and non-additive genetic effects in that differentiation and if negative genetic interactions are involved (Chapter 2). These questions are relevant in terms of Evolutionary Biology, particularly understanding the speciation process originating these species. Reproductive isolation is an important part of speciation and there are many kinds of reproductive barriers, assortative mating being one important prezygotic barrier. In Chapter 3, I provide the results of assays performed to determine if *D. madeirensis* and *D. subobscura* have assortative mating and what are the consequences, if any, of mating with heterospecifics in terms of fecundity. Another interesting issue relates with developmental problems in hybrids. Generally, interspecific hybrids present several problems, the most extreme being sterility and inviability. However they can also present other less drastic effects, like developmental problems resulting in morphological abnormalities that can reduce hybrid fitness. Having this in mind, the third issue I addressed in this thesis was if interspecific hybridization increased

fluctuating asymmetry (a measure of developmental stability) and developmental noise (Chapter 4). The issues developed in Chapters 3 and 4 are particularly important in terms of speciation but also for understanding the buffering mechanisms controlling development (Chapter 4). Finally, in Chapter 5, I tried to answer the question: do different closely related species converge under similar selective pressures? This is an important issue not only in terms of Evolutionary Biology, pertaining with the implications that different genetic backgrounds may have in population/species evolution, but also in terms of Conservation, namely for captive breeding programs. In the following sections I will address the results obtained and analyse their contribution to these Evolutionary Biology, Speciation, Development and Conservation issues. I will end with some general remarks and possible future directions for further work.

6.1. Species differentiation –The role of additive and non-additive genetic effects

Drosophila madeirensis and *Drosophila subobscura* are clearly differentiated in terms of several life history traits. The results indicate that in general *D. subobscura* has a better performance, especially in fecundity, than *D. madeirensis*. The observed differentiation involves both additive and non-additive genetic effects, namely negative dominance and epistasis (Rego *et al.*, 2007a, Chapter 2). Dominance is often associated with population differentiation (e.g. Lynch and Walsh, 1998), however, the evidences point mainly to positive dominance, heterosis being frequently observed (e.g. Edmands, 1999; Fenster and Galloway, 2000; Bieri and Kawecki, 2003; Facon *et al*., 2005). Comparatively, evidences of negative dominance are scarcer (Teotónio *et al*., 2004).

The finding of negative epistasis is important in terms of the standing controversy regarding the role of epistasis in population differentiation. This controversy dates back to two of the most influential contributors to the neoDarwinian synthesis: Fisher and Wright. These authors disagreed on the relevance that gene interaction may have in population differentiation. Wright proposed the Shifting Balance theory where epistasis played a fundamental role in population divergence, while Fisher considered epistasis irrelevant and believed that additive effects were sufficient to explain population evolution. The finding of significant negative epistasis

in the differentiation of closely related species is thus extremely important and lends some support to Wright's point of view.

However, the finding of negative dominance and epistasis on present differentiation between our species does not prove that these effects played a significant role in the divergence process involved in their speciation (Coyne, 1992; Fenster *et al.*, 1997). Namely it is not clear if gene interaction was a cause or a consequence of the divergence process (Fenster *et al.*, 1997; Johnson, 2002). Recently the possible importance of epistasis to speciation has been supported by several authors, namely in shaping reproductive isolation mechanisms, an important part of this process (e.g. Turelli and Orr, 2000; Wade, 2002). Also important is to explore in more detail the role of negative dominance in species differentiation, an effect with similar evolutionary implications, but generally overlooked in most studies. In spite the mentioned limitations, our findings of genetic constraints expressed by negative non-additive genetic effects in the differentiation between closely related species, are important and reinforce the need for further studies in this area. Namely it will be important to analyse if these constraints are related with the speciation process directly and/or if they contribute to sustained reproductive barriers,

We should also mention that one of the main reasons for the scarcity of interspecific line-cross studies is the difficulty in obtaining the necessary hybrid generations: e.g. F_1, F_2 and backcrosses. In this respect, we would like to point out that the ability to obtain hybrid generations could be influenced by the number of founders and maintenance regime of the parental base stocks. The *D. madeirensis* and *D. subobscura* populations used in this work were founded using a large number of individuals and were maintained in outbred conditions with a relatively large effective size. On the other hand, previous studies analysing these species and their hybrids used isofemale lines, most probably with a low genetic variability. This could be the reason why these previous studies concluded that F_1 male hybrids between these species are sterile (e.g. Krimbas and Loukas, 1984; Khadem and Krimbas, 1991, 1993; Papaceit *et al.*, 1991), and we obtained the opposite result.

6.2. Assortative mating - does it act as a reproductive barrier?

Reproductive isolation plays an important role in species differentiation. *Drosophila madeirensis* and *D. subobscura* show positive assortative mating, conspecific matings being more probable than heterospecific ones. Moreover, the heterospecific cross involving *D. subobscura* females occurs more frequently than the reciprocal one (Rego *et al*., 2007c, Chapter 3). The difficulties observed in the cross between *D. madeirensis* females and *D. subobscura* males suggest a (incomplete) prezygotic reproductive barrier.

These observations, together with the analysis of the different fecundities presented by females mated with heterospecific *vs.* conspecific males, indicate that the reproductive barriers preventing hybridization between *Drosophila madeirensis* and *D. subobscura* are different in the two cross directions (Rego *et al*., 2007c). In the direction involving *D. subobscura* females and *D. madeirensis* males, the barrier seems to be mainly postzygotic. This heterospecific mating is relatively easy to observe in behavioural assays; however, the ensuing hybrid offspring presents a biased sex-ratio strongly favouring males and produces fewer hybrids. On the other hand, matings from the reciprocal cross are much harder to observe, but when they occur they produce a relatively high number of offspring with an even sex-ratio, suggesting that in this case the reproductive barrier is prezygotic (Rego *et al.*, 2007a, c; Chapters 3 and 4).

What is the relevance of this finding in speciation terms? As stated before, reproductive isolation is an important part of the speciation process, and the expectation of the evolution of postzygotic reproductive barriers follows a given pattern in accordance with Haldane's rule. According with this rule, the first reproductive barrier to evolve is hybrid male sterility followed by hybrid male inviability, females being affected last, first by sterility and later by inviability (Coyne and Orr, 1989, 1997). Our findings are in contradiction with this pattern. The relative inviability of female hybrids from the cross involving *D. subobscura* females is thus an interesting finding because it contradicts one of the most pervasive tenets in Evolutionary Biology, one that has normally been confirmed in *Drosophila* species.

Why are hybrid females from this cross inviable? Why do the two heterospecific cross directions suggest different reproductive barriers? Asymmetry in assortative mating, i.e. one heterospecific cross direction being easier to observe than the reciprocal

one, has been a common finding in *Drosophila* species (Ödeen and Florin, 2002), and has even been related with the direction of evolution (Kaneshiro, 1976, 1980; Watanabe and Kawanishi, 1979). However, to our knowledge, this is the first time an asymmetry involving pre- and postzygotic barriers between reciprocal cross directions is described. In terms of the speciation process what could be the implications of this asymmetry? Generally speaking, this would mean that the facilitated route by which F_1 hybrids could influence the evolution of these species, would be through backcrossing with hybrids with *D. subobscura* maternal origin. However, to ascertain the implications of hybridization in the future evolution of these species it would be necessary to determine the frequency of natural hybrids and backcrosses, as well as the degree of introgression between *D. madeirensis* and *D. subobscura*. The fact that *D. madeirensis* is an endemic species associated with a threatened habitat makes this question even more relevant, given that habitat fragmentation and general changes of landscape can increase the chances for hybridization to occur (Rhymer and Simberloff, 1996; Allendorf *et al.*, 2001) .

6.3. Developmental stability – Is developmental noise higher in hybrids?

It is generally assumed that hybridization has a detrimental effect in the mechanisms responsible for buffering development against perturbations (Alibert and Auffray, 2003). The results of our analysis comparing developmental stability between F_1 hybrids and parental species, challenge this believe, as they indicate that although female F_1 hybrids, from the cross direction with *D. madeirensis* mothers, present increased levels of wing size bilateral asymmetry when compared to parental species, this asymmetry does not reflect higher developmental noise (Rego *et al.*, 2006, Chapter 4). On the other hand, hybrid males from both cross directions, present similar asymmetry levels to parental species.

The results also suggest that the mechanisms underlying canalization and developmental stability are somewhat independent, as indicated by the low congruence between the covariation structures of the interindividual genetic components (a measure of canalization) and the intraindividual ones (a measure of developmental stability). This is in accordance with previous findings on mouse craniums (Debat *et al.*, 2000)

and contradicts the notion defended by several authors that developmental stability is a particular case of canalization (e.g. Clarke, 1998; Klingenberg and McIntyre, 1998; Meiklejohn and Hartl, 2002). This controversy raises the need for further studies to determine in which contexts (traits, genetic backgrounds) canalization and developmental stability are determined independently and in which they are not.

Interestingly enough, hybrid females are once again the more affected sex, which is another example from our work that contradicts Haldane's rule (see above, Rego *et al.*, 2006, 2007a). A possible explanation for the observed differences between sexes, may be related with recent findings on differential gene expression comparing hybrids with parental species. Several studies comparing gene expression patterns between parental species and hybrids in *Drosophila*, showed that hybrids tended to misexpress several genes, overexpressing or underexpressing them, and linked this misexpression with hybrid dysfunctions (Michalak and Noor, 2003; Ranz *et al.*, 2004; Noor, 2005; Hearty and Singh, 2007; Moehring *et al.*, 2007). Some studies also indicate that this effect is higher in hybrid females (e.g. Michalak and Noor, 2003; Ranz *et al.*, 2004; Noor, 2005), which could be related with a higher asymmetry as presented here.

As mentioned above, our data suggest that the disruption of symmetry-generating mechanisms due to hybridization may be responsible for increased asymmetry in hybrids rather than increased developmental noise. It would be very interesting to determine if gene misexpression is somewhat related with developmental buffering mechanisms. Asymmetry levels are often associated with decreased fitness (Moller, 1993; Palmer, 1994), and in our case some hybrid females exhibit a huge asymmetry, one that in some cases could even compromise proper flying capability (Rego *et al.*, 2006, Chapter 4). Considering that hybridization does occur in nature and follows the same patterns we observed in the lab, which may not always be the case (Llopart *et al*., 2005); then, if behavioral barriers are somewhat overcome in nature, F_1 hybrids with *D. madeirensis* mothers could be "influential" in determining the consequences of hybridization for the evolution of these species. However, the high asymmetry presented by female hybrids could reduce their fitness, acting as a potential post-zygotic barrier, and consequently, reducing their impact in the evolution of *D. madeirensis* and *D. subobscura*. This would mean that hybrid males, particularly the ones with *D. subobscura* mothers, would be potentially more influential in the future evolution of these species.

6.4. Adaptive potential - Do different species converge when placed under similar conditions?

Our results, comparing the initial differentiation and evolutionary trajectories of *D. madeirensis* and *D. subobscura* populations, during adaptation to captivity over several generations, indicate that, in spite of being very closely related, they differed in their capacity to adapt to the novel, common environment (Rego *et al.*, 2007c, Chapter 5). As we have seen in previously mentioned studies, both species are clearly differentiated in terms of life history traits, *D. subobscura* presenting a better performance in most traits (see above, Rego *et al.*, 2007a, b, Chapters 2 and 5). The analysis of evolutionary trajectories indicates that in spite of both species showing signs of improvement in the new environment (adaptation), particularly in age of first reproduction and early fecundity, they differed in their adaptive response, with *D. subobscura* presenting a clear pattern and a higher rate of improvement. The observed differences in "adaptive potential" could be related with different ecological requirements. *D. subobscura* is a generalist species with a wide geographic distribution, and thus presumably is better equipped to deal with new selective pressures including captivity (Parsons, 1982). On the other hand, *D. madeirensis* is an endemic species with a restricted distribution, specialized in the resources provided by the Laurisilva forest. Moreover, this species is also monomorphic for chromosomal inversions (Khadem and Krimbas, 1993), a possible indicator of low genetic variability. Some authors have suggested that different genetic backgrounds may influence the outcome of the natural expectation of convergence under similar selective pressures (Cohan, 1984a, b; Cohan and Hoffmann, 1989). Our data support this view and are in accordance with previous findings in intraspecific studies (Matos *et al*., 2002; Simões *et al*., 2007a, b).

These findings have important repercussions not only in terms of Evolutionary Biology, but also for Conservation issues. Nowadays, many species are involved in captive breeding programs (*ex-situ* conservation programs) to ensure any chance for future survival. Unfortunately, it is expected that in the near future, many more will need this kind of intervention (e.g. Soulé *et al*., 1986; Tudge, 1995), due to habitat fragmentation and destruction. Consequently, it is crucial that we understand what happens in evolutionary terms to captive populations. It is important to consider that different genetic backgrounds can play a decisive role in the way species "adapt" (or

not) in general, and to captivity in particular. From previous studies, we already know that the genetic changes induced by adaptation to captivity can be detrimental for future reintroductions (Woodworth *et al.*, 2002). Nevertheless, it is also a requirement that populations are maintained with acceptable large population sizes, to minimize loss of genetic variability and inbreeding depression. While it is not consensual how to manage populations in *ex-situ* conservation programmes, in our opinion, in face of these problems, long term maintenance may be more detrimental to populations having lower performance during the captive period. Our data indicate that different species, even closely related ones like *Drosophila madeirensis* and *D. subobscura*, may react differently to captivity, even failing to adapt to the new conditions. This can pose additional problems to captive breeding programs and reintroduction, and leads to a word of caution in generalizations of management procedures between species, even if they are closely related (as ours). This raises the need for further studies involving other organisms, particularly species with different ecological requirements, other than the cosmopolitan generalist species *D. melanogaster*, the still traditional model organism in Evolutionary Biology.

6.5. Final remarks and future directions

The larger the island of knowledge, the longer the shoreline of wonder.

Ralph Sockman

When I started this project I had many questions I wanted to answer, but, as it happens in most cases, the answers I got aroused new questions.

Negative epistasis is fundamental to the fitness landscape proposed by Wright and his Shifting Balance theory (Wright, 1977). Our results presented in Chapter 2 are important as they pertain to the way populations may cross fitness valleys and warrant further research, particularly in determining which of the three types of gene interaction (additive x additive, additive x dominance and dominance x dominance) are more important and what are the signs of these interactions. As we stated before (Rego *et al.*, 2007a) our analysis did not allow the partition of epistatic effects in all possible digenic interactions. Consequently, we only looked at epistasis as a whole, meaning that it is

possible that the several gene interactions present different signs, involving different evolutionary implications. To clarify this issue it will be necessary to conduct a line-cross analysis using Mather and Jinks (1982) coefficients, comparing not only parental, F_1 and F_2 hybrids but also backcrosses from both cross directions (Kearsey and Pooni, 1996). This analysis should also be extended to other species pairs to find out if the observed patterns can be generalized. As one of the major limitations in this field is obtaining the necessary hybrid generations to conduct these assays, it would be interesting to explore other species pairs with similar divergence times (0.6 - 1 Myr, Ramos-Onsins *et al*., 1998), to see if the observed patterns can be generalized.

The results obtained in Chapter 3, concerning behaviour and fecundity assays and reproductive isolation raise an important question: why is it so hard to observe heterogamic matings involving *Drosophila madeirensis* females? Given that this is the most prolific cross in terms of hybrids, this is in fact an intriguing question which warrants further investigation. Namely, it would be interesting to determine if it is due to female discrimination, male lack of interest or a combination of both. Another interesting question would be to compare results using choice and no choice tests to analyse the influence of competition between males, this would enable us to determine if *D. madeirensis* males are indiscriminate when given the choice to court conspecific and heterospecific females.

An additional important issue raised by our data is the observed asymmetry in reproductive barriers between reciprocal cross directions; this warrants more studies to determine if other species pairs present similar asymmetries and to determine the implications of this asymmetry to the speciation process. Also, as the results from studies of hybridization may be decoupled between a laboratorial environment and what 'really' happens in the natural habitat, it would be interesting to determine and measure natural hybridization and eventual introgression between these species. Such studies are particularly important given the ecological-evolutionary context involved between *D. madeirensis* and *D. subobscura*, two closely related co-occurring species, one of them being an endemic species associated with a threatened habitat.

Our results on hybrid developmental stability call for similar studies to verify if this pattern allows further generalization. For example, it would be interesting to complement this study with data on female hybrids with *D. subobscura* mothers, and given that F_1 hybrids from both cross directions are relatively fertile, it would also be

interesting to analyse what happens to F_2 hybrids in terms of asymmetry patterns, will they express additivity, similarity to parental species or increased asymmetry due to hybrid breakdown? An additional appealing area to investigate is to analyse other species pairs, including species with varying divergence times, to test for generalizations of our finding that increased asymmetry in hybrids is not due to developmental noise, and to see if the result is influenced by the amount of differentiation between species as suggested by Markow and Ricker (1991).

Another promising venue for future research in this area would be analysing the relationship of differential gene expression in hybrids and parental species, in what concerns developmental buffering mechanisms and asymmetry patterns. Owing to the fact that female hybrids tend to express higher asymmetry, one such study would be comparing both sexes in terms of gene expression related with candidate genes involved in wing development and buffering mechanisms.

Finally our analysis on evolutionary trajectories comparing *D. madeirensis* and *D. subobscura* indicated that differences in genetic backgrounds may have a say in the way species adapt to new conditions. This calls for more studies to check for repeatability of results, including more detailed and prolonged evolutionary trajectories, to characterize more accurately the evolutionary response during adaptation. Further studies should also analyse several populations independently derived from Madeira, as well as other species to determine if the differences are at the species level and not due to effects of foundation. For generalization purposes, it would be particularly interesting to include species with different ecological requirements, e.g. generalists *vs.* specialists.

All these additional studies will further our knowledge in several fields, ranging from Speciation, Evolutionary Biology to Development and Conservation, never forgetting that: *The larger the island of knowledge, the longer the shoreline of wonder* (Ralph Sockman).

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