EVOLUTIONARY GENOMICS FROM ONTOGENY TO PHYLOGENY

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FROM ONTOGENY TO PHYLOGENY

By

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A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

McMaster University

DOCTOR OF PHILOSOPHY (2009) McMASTER UNIVERSITY

(Biology) Hamilton, Ontario

TITLE: Evolutionary Genomics from Ontogeny to Phylogeny

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NUMBER OF PAGES: [xii], 173

ABSTRACT

Much speculation has been made about the relative importance of changes in developmental regulation of gene expression in determining major phylogenetic patterns observed both in extant and extinct species. However, most of these hypotheses have been formulated based on data obtained from the comparison of very distantly related organisms (e.g., between animal phyla). Another approach to answering questions about development (ontogeny) in the context of evolution (phylogeny) is to observe how developmental patterns diverge between closely related species, in order to obtain a better understanding of the population level processes underlying phyletic change. With the intent of addressing this possibility, the principle work outlined in this thesis investigated patterns of divergence between closely related species of *Drosophila* at the level of both the nucleotide coding sequence as well as gene expression levels in the context of ontogeny. The results show that the stage during which genes are expressed has a significant impact on their patterns of divergence, acting both to constrain (earlier stages) and accelerate (later stages) their rates of evolution - the latter being largely the result of sexual selection pressure. However, we also find that intermediate stages of fly development, such as metamorphosis, may experience a greater degree of conservation of the elements regulating gene expression than other stages. Nonetheless, we do find evidence that both gene expression and coding sequences may be subject to similar selection pressures, yet there also appears to be substantial uncoupling of the two, as evidenced by our observation of stage-specific, autonomous patterns of hybrid misexpression manifested in interspecific hybrids. The data presented herein shed new light on patterns of divergence between species, specifically with regards to how various selection pressures affect different stages of ontogeny.

ACKNOWLEDGEMENTS

John Dewey (1859 – 1952), American philosopher, psychologist, educational reformer, and lapsed Haeckelian, held views much in line with W.B. Yeats' old aphorism about likening education to the lighting of a fire, rather than the filling a bucket, when he wrote:

> "Knowledge," in the sense of information, means the working capital, the indispensable resources, of further inquiry; of finding out, or learning, more things. Frequently it is treated as an end in itself, and then the goal becomes to heap it up and display it when called for. This static, cold-storage ideal of knowledge is inimical to educative development. It not only lets occasions for thinking go unused, but it swamps thinking. No one could construct a house on ground cluttered with miscellaneous junk. Pupils who have stored their "minds" with all kinds of material which they have never put to intellectual uses are sure to be hampered when they try to think. They have no practice in selecting what is appropriate, and no criterion to go by; everything is on the same dead static level."
>
> -John Dewey, *Democracy and Education* (1916)

I can think of no quotation that more fittingly describes the contributions to my personal development made by my supervisor, Dr. Rama Singh. While some students may prefer the reassurance provided by a 'full bucket', I've come to realize that Dr. Singh's philosophy of self-sufficiency (served with a healthy dose of encouragement and work-related discussion at the Phoenix Pub or Faculty Club), has provided me with the tools that I will require to continue my career in the often stressful, endlessly competitive, but ultimately rewarding field of scientific research. For such a boon, I shall remain eternally grateful.

An equal amount of thanks must go to Dr. Wilfried Haerty, a former post-doctoral fellow in the Singh lab, who acted both in the capacity of a co-supervisor and a good friend. Much of the work that I've accomplished over the course of my education here at McMaster was made possible by his helpful advice and endless patience. I am also grateful to the members of the faculty of Biology who made up my supervisory committee, Drs. Brian Golding, Ana Campos, Ben Evans, and Richard Morton, for their constructive criticism and helpful advice. Science is a collaborative endeavor, and their friendly, knowledgeable aid has helped immensely.

Speaking of collaborations, thanks must also go to the other members of the Singh lab, both current and former, especially Santosh Jagadeeshan and Abha Ahuja who provided both constructive criticism and helpful encouragement during all of the myriad

challenges I faced in completing my work. Such appreciation must also be extended to my peers in graduate studies, notably Maria Abou-Chakra, David Anderson, Adam Bewick, Steven Brown, Freddy Chain, Paul Craig, Weilong Hao, Melanie (Sr.) Huntley, Melanie (Jr.) Lou, Allyson MacLean, Bart Maslikowski, Andrea Morash, Luca Poloni, Marie-Pierre Schippers, Iqbal Setiadi, and Wilson Sung. Thanks for making the department of biology such a great working environment.

On the technical side, I must thank Dr. Timothy Westwood, and his technician Kaiguo Mo at the Canadian *Drosophila* Microarray Center for the immensity of their assistance with regards to the gene expression work that I've performed. I must also thank Dr. Grant McClelland, and again his student Paul Craig, for allowing me to use their qPCR equipment, in addition to instructing me in its proper use. I would also like to acknowledge the assistance of three excellent undergraduates: Mariša Melas, Priya Iyer, and Marek Gruca.

Special thanks must be reserved for my father Emidio, my mother Joanne, my sister Nadia, and the other members of my family for their love and encouragement. And finally, most of all, to my girlfriend Julie for putting up with all of the stress that I'd occasionally bring home from the lab. Her love and support kept my 'head above the water' most of all.

The dedication to my M.Sc. thesis read: "To my high-school principle, who told me that I was not 'university material'. Thank you for giving me a goal to pursue and thus the drive to succeed." I shall not break with tradition, and again dedicate my Ph.D. thesis to the same individual. As a man who dedicated his own life to education, I'm certain that he'll be happy to learn that, given the existence of this manuscript, his hypothesis about my composition likely warrants rejection.

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CHAPTER 1

GENERAL INTRODUCTION

"I also believe that an understanding of regulation must lie at the center of any rapprochement between molecular and evolutionary biology; for a synthesis of the two biologies will surely take place, if it occurs at all, on the common field of development."

-Stephen Jay Gould, *Ontogeny and Phylogeny* (1977)

1.1 Overview

The establishment of a new field of study or avenue of research is often burdened by the weight of expectation that its discoveries will offer revolutionary insight into questions that have thus far remained unanswered (Hoekstra and Coyne 2007). In some cases such insight is obtained, such as when G.A. Parker (1970) made the realization that intrasexual male-male competition could continue beyond the moment of copulation in the form of sperm competition. This insight preceded a renewal of interest in the field of sexual selection, and produced many testable predictions that were subsequently confirmed (see Andersson 1994; Arnqvist and Rowe 2004 for review). On the other hand, other original avenues of research, such as Eldredge and Gould's (1972) theory of punctuated equilibrium, have produced results that are far more equivocal, despite the initial optimism under which their theoretical underpinnings were introduced (Hoekstra and Coyne 2007). Within the broad umbrella of the biological sciences, evolutionary developmental biology, or 'evo-devo' - an attempt to synthesize the once disparate fields of developmental biology (ontogeny) and evolutionary biology (phylogeny) - has relatively recently begun to publicize the discovery of important insights into how macro-evolutionary processes occur (see Carroll 2005a,b; 2008; Davidson and Erwin 2006; Wray 2007, for example). Specifically, proponents of evo-devo have argued that there exists a fundamental dichotomy between the effects of mutations occurring at loci involved in morphological development and those involved in 'physiological processes' (which includes everything not involved in morphological development [Hoekstra and Coyne 2007]). Genes within the former category are said to be involved in a greater degree of pleiotropy resulting from interconnectivity within pathways regulating the developmental process, and are thus more likely to produce deleterious fitness consequences when substitutions occur within their protein coding sequences (Carroll 2005a,b). In order to obviate these consequences, members of the evo-devo community

have argued that changes in *cis* regulatory elements (sequences of DNA that regulate the expression of genes located on the same nucleotide strand) of genes involved in complex developmental pathways are the predominant mechanism through which evolution has shaped morphology, as the modular nature of these elements allows them to regulate gene expression in a tissue- and ontgenic stage-specific manner (reviewed in Carroll 2005a, 2008).

While the hypothesis that *cis* regulatory elements, and consequently variation in patterns of gene expression, have played a disproportionate role in the evolution of animal form is both insightful as well as testable, what data do exist on the subject typically concern studies involving the investigation of individual genes, leaving the broader genome-level, phylogenetic patterns upon which the hypothesis will be confirmed or rejected relatively unexplored (Hoekstra and Coyne 2007). Nonetheless, this scarcity of data has not prevented copious speculation about the importance of the evolution of developmental regulation in charting the course of evolutionary history. Such speculation has occasionally gone so far as to deride the fundamental innovations gleaned from decades of work conducted in more traditional sub disciplines of evolutionary biology altogether (e.g., population genetics), suggesting that we must abandon previously fruitful avenues of research (or at the very least, modify them substantially):

> The homologies of process within morphogenetic fields provide some of the best evidence for evolution – just as skeletal and organ homologies did earlier. Thus the evidence for evolution is better than ever. The role of natural selection in evolution, however, is seen to play less an important role. It is merely a filter for unsuccessful morphologies generated by development. Population genetics is destined to change if it is not to become as irrelevant to evolution as Newtonian mechanics is to contemporary physics. (Gilbert et al. 1996, 368)

Lest the above quotation be considered an isolated instance, such views have recently been championed in the work of Davidson and Erwin (2006) who have claimed that "classic evolutionary theory, based on selection of small incremental changes" cannot provide "an explanation of evolution in terms of mechanistic changes in the genetic regulatory program for the development of the body plan" (Davidson and Erwin 2006, 796; but see also Coyne 2006 for criticism). Evo-devo, for the most part, remains firmly a product of the discipline of developmental genetics from whence it originated (Gilbert et al. 1996), with an emphasis on phenomenological descriptions of conserved processes among widely divergent taxa rather than the development of a rigorous mechanistic theory. Unbridled speculation coupled to a lack of theoretical underpinning has led to a widening gulf between scientists enthusiastically pursuing the current formulation of evo-devo, and those calling for a more thorough integration of principles derived from molecular evolution and population genetics in understanding the importance played by the evolution of development in phylogenetic history (Stern 2000; Johnson and Porter

2001; Wilkins 2002; Coyne 2006).

Johnson and Porter (2001) have advocated an approach to reconciling modern evo-devo with evolutionary biology that avoids 'throwing the baby out with the bathwater', so to speak. They suggest that the climax of the synthesis between ontogeny and phylogeny will occur when population genetics properly incorporates knowledge gained from the study of development:

> A centerpiece of this synthesis of population genetics and development will be a mechanistic theory of adaptation, one rooted in what we know about how phenotypes arise from genotypes. [...] Such a theory would also advance the concept of developmental constraint in evolutionary biology, making it mechanistic rather than strictly phenomenological. (Johnson and Porter 2001, 55)

While in the past, evo-devo has generally been primarily concerned with explaining how similar developmental programs have been conserved among very divergent taxa (e.g., the homeobox, or HOX proteins that underlie anterior-posterior patterning in the vast majority of studied animals [Lewis 1978; Nusslelein-Volhard and Weischaus 1980; see Johnson and Porter 2001 for review]), the aforementioned synthesis will hinge upon developmental and regulatory comparisons among very closely related species, where researchers can obtain a better understanding of the population processes that bring about interspecific differences; or what Johnson (2007) calls 'the micro-evolution of development'. Johnson and Porter (2001) have noted that the study of speciation has been viewed as the 'methodological bridge' uniting theories of micro- and macro-evolution (e.g., Futuyma 1998, Wade 2000). Furthermore, an important aspect of speciation research, the study of reproductive isolation via observation of the phenotypes of interspecific hybrids, has often found that such hybrids manifest varying degrees of breakdown of proper organogenesis and other developmental systems (see Coyne and Orr 2004; Ortíz-Barrientos et al. 2007); thus Johnson and Porter (2001, 52) "believe that the investigation of speciation is... a logical place to build a bridge between population genetic and evolution-of-development studies."

The overall goal of the material presented herein is to contribute to the ongoing effort to complete Gould's (1977) 'rapprochement' between ontogeny and phylogeny, from the perspective of the field of evolutionary biology. Building upon the suggestions discussed above, I have sought to study patterns of speciation with a special focus on development under the expectation that the patterns I observe will offer insight into how developmental-specific processes diverge between closely-related species. As will be discussed below, sexual selection plays a prominent role in both aforementioned processes and thus has also acted as a significant component of this research. As the experimental approaches detailed in the chapters that constitute this work cover a broad sample of past as well as ongoing research in both ontogeny and phylogeny, I shall begin

with a brief review of the pertinent concepts in the fields of speciation genetics (§ 1.2) and the evolution of development (§ 1.3).

1.2 Speciation Genetics

> "He who explains the generation of species through purely natural agencies should assign a natural cause for this remarkable result; and this Mr. Darwin has not done."
>
> -Asa Gray, *The Origin of Species by Means of Natural Selection* (1860, 51)

1.2.1 The genetic 'problem' of speciation

In what must be considered one of the greatest 'titular' ironies, Darwin's *On the Origin of Species* failed to present a natural mechanism by which new species are formed (Darwin 1859; Orr 1996a; Coyne and Orr 2004). This is to say, how two populations, originally readily able to interbreed, could diverge independently such that mechanisms preventing their fusion could arise (Dobzhansky 1937; Mayr 1970). Such 'reproductive isolation' mechanisms are broadly classified into two categories: those that reduce the probability of formation of hybrid zygotes, such as geographic isolation or behavioral divergence (termed 'pre-zygotic'), and those that reduce the fertility of hybrid zygotes (termed 'post-zygotic'). Because of its bearing on the work presented in this thesis, this discussion will focus primarily on the latter mechanism. Though Darwin never provided a mechanism through which speciation could occur, he did include an entire chapter on interpopulation and interspecific hybridization, wherein he made an excellent argument for why we should expect post-zygotic reproductive isolation to have occurred by natural means rather than by the then prevailing view that "species, when intercrossed, have been specially endowed with sterility, in order to prevent the confusion of all organic forms." (Darwin 1859, 474). If hybrid sterility had been 'designed' in order to maintain the purity of species, he reasoned, it would not explain why we observe varying degrees of sterility or inviability in hybrids, depending on such factors as morphological similarity between parents, and the direction of the cross (i.e., which parent is the female and which is the male):

> For why should the sterility be so extremely different in degree, when various species are crossed, all of which we must suppose it would be equally important to keep from blending together? (Darwin 1859, 481)

Unfortunately, it was difficult to envision a situation by which natural selection could favor hybrid sterility or inviability, as both of these situations are patently

maladaptive. Darwin nonetheless made the intuitive suggestion that sterility in hybrids likely arises because, in mixing parental genotypes "the organisation [has] been disturbed by two organisations having been compounded into one" (Darwin 1859, 485). Or, when translated into modern language, he realized that independent genetic divergence between isolated populations would eventually lead to incompatibilities that either reduced the fitness of hybrids, or prevented fertilization in the first place. It should be noted that I will define separate species via the 'biological species concept' or BSC, first introduced by Ernst Mayr: "species are groups of interbreeding natural populations that are reproductively isolated from other such groups," (Mayr 1942, 1996; Coyne and Orr 2004). While the BSC has been criticized for both not being universally applicable (it requires sexual reproduction, for instance), nor taking phylogenetic history into account (e.g., Velasco 2008), it remains the most widely used species definition, and its opponents nonetheless recognize its significance in the creation of biodiversity (Johnson 2000; Coyne and Orr 2004).

While insightful, the notion that dysfunction in hybrids arose because of incompatible genetic divergence between populations was initially met with considerable skepticism and theoretical difficulty (Orr 1996a). In order to illustrate why this was the case, imagine that two populations reside on separate adaptive 'peaks' (using Wrightian language) and are separated by a fitness 'valley' representing the genotype of their hybrids. The fundamental problem of speciation thus lies in explaining how each species was able to arrive at its own peak without having had to cross through the valley, which for many hybrids represents complete sterility or inviability, i.e., a fitness of zero (Wright 1932; Orr 1997). Put into simple genetic terms, imagine species 1 having genotype A_1A_1 and species 2 having genotype A_2A_2 (I use subscripts to designate alleles in order to avoid considerations of dominance and recessiveness). Consequently, A_1A_2 represents the lower fitness hybrid genotype. Given that mutations always arise in a heterozygous state, if A_1A_1 was the ancestral state, then species 2 could not have arrived at its genotype without having passed through the unfit hybrid genotype A_1A_2, which should be subject to purifying selection (Muller 1940; Orr 1996a) (Figure 1.1A).

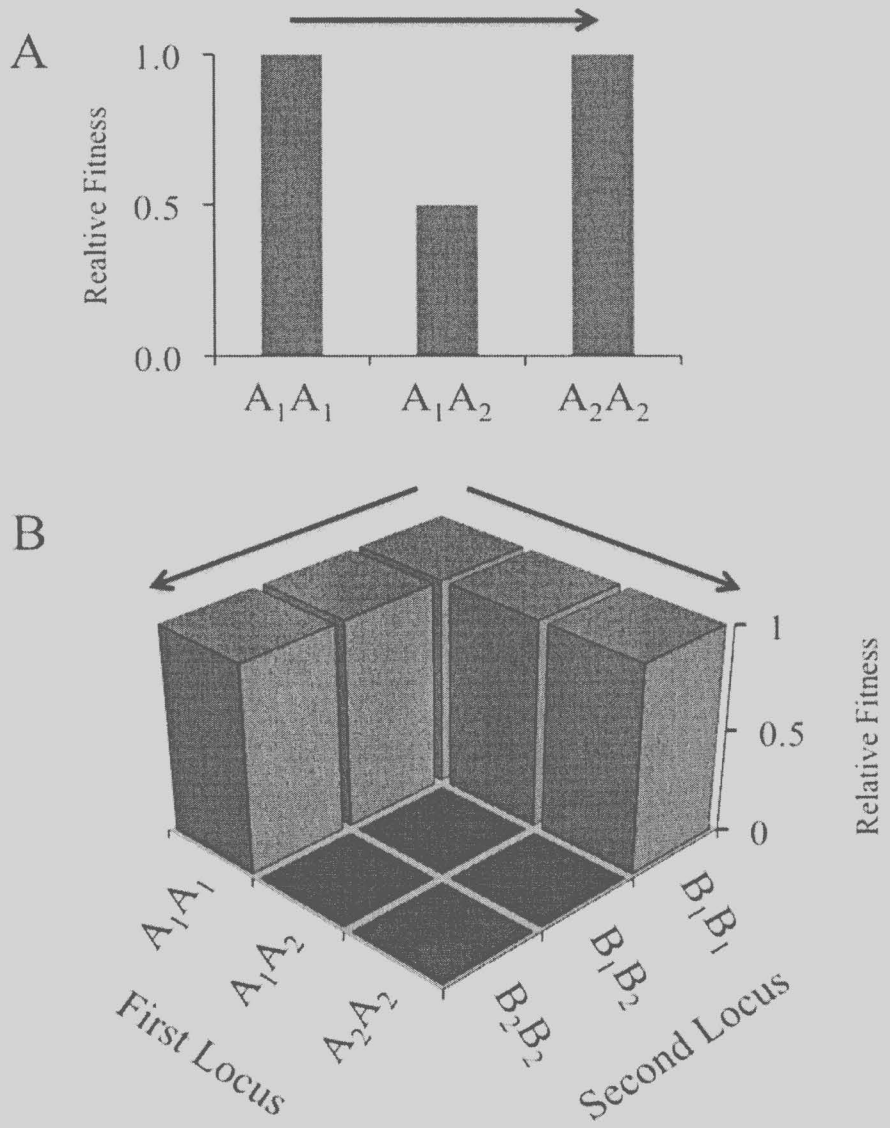

Figure 1.1: Graphical illustration of the one-locus (A) and two-locus or 'Bateson-Dobzhansky-Muller' (B) models of genetic hybrid incompatibility. The height of a bar indicates the corresponding relative fitness of that genotype. Note that in the one locus model, evolution from one genotype to another (A_1A_1 to A_2A_2), represented by the arrow, requires passing through the unfit hybrid genotype wherein alleles A_1 and A_2 are incompatible. In the case of the two-locus model, it is possible to reach the descendent species' genotypes ($A_2A_2B_1B_1$ and $A_1A_1B_2B_2$) without crossing through an unfit hybrid genotype consisting of the incompatible combination of the A_2 and B_2 alleles. The relative hybrid genotype fitnesses are arbitrary, though in the case of the two-locus model a hybrid fitness of zero was chosen (i.e., complete hybrid sterility) for clarity of illustration. Adapted from Gavrilets 2003.

It is now recognized that William Bateson first presented the solution as to how species could cross through the hybrid valley in an obscure paper published in 1909 that failed to garner much attention from the speciation community (Bateson 1909; see Orr 1996a):

> Now if the sterility of the cross-bred be really the consequence of the meeting of two complementary factors, we see that the phenomenon could only be produced among the divergent offspring of one species by the acquisition of at least two new factors; for if the acquisition of a single factor caused sterility the line would then end. Moreover each factor must be separately acquired by distinct individuals, for if both were present together, the possessors would by hypothesis be sterile. And in order to imitate the case of species each of these factors must be acquired by distinct breeds. The factors need not, and probably would not, produce any other perceptible effects; they might, like the colour-factors present in white flowers, make no difference in the form or other characters. Not till the cross was actually made between the two complementary individuals would either factor come into play, and the effects even then might be unobserved until an attempt was made to breed from the cross-bred. (Bateson 1909, 81)

Bateson recognized that the difficulty of passing through the hybrid genotype could be overcome if sterility was not the result of incompatibility between alleles at a single locus, but rather due to the incompatible interaction of alleles at two (or more) loci, which he called 'complimentary factors'. Again, in the simplest genetic example, imagine that one population is of genotype $A_1A_1B_2B_2$ and a second population of genotype $A_2A_2B_1B_1$, with their hybrids thus being of genotype $A_1A_2B_1B_2$. If A and B are interacting loci, it is conceivable that while the A_1-B_2 and A_2-B_1 combinations are capable of functional interaction, the A_2 and B_2 alleles are incompatible and thus produce a sterile phenotype in the hybrids (these alleles have never 'seen' each other in natural populations and thus have not been selected for compatibility [Coyne and Orr 2004]). What is crucial to this model is that, assuming for example, that $A_1A_1B_1B_1$ is the ancestral genotype, it is possible to arrive at the two derived genotypes without ever having passed through the incompatible $A_1A_2B_1B_2$ genotype: $A_1A_1B_1B_1 \Rightarrow A_1A_2B_1B_1 \Rightarrow A_2A_2B_1B_1$, and similarly $A_1A_1B_1B_1 \Rightarrow A_1A_1B_2B_1 \Rightarrow A_1A_1B_2B_2$ (Figure 1.1B; Coyne and Orr 2004).

Though evidently unaware of Bateson's work, it was Theodosius Dobzhansky who first provided empirical evidence that incompatible interactions among loci lay at the basis of hybrid sterility when he showed that the sterility of backcross hybrids between *Drosophila pseudoobscura* and *D. persimilis* (< 1 MYD; Tamura et al. 2004) resulted from the interaction of loci within and between chromosomes (Dobzhansky 1936; Orr 1996a). This classic study also had the benefit of putting to rest alternative hypotheses about the cause(s) of hybrid sterility, such as the relatively popular idea that sterility was

caused via the interaction of genetic and cytoplasmic elements: Dobzhansky found that backcrosses transferring all of one species' chromosomes into another's cytoplasm were fertile (Orr 1996a). A second alternative hypothesis suggested that the most common mechanism of speciation involved independently fixed chromosomal rearrangements, such that chromosomes failed to pair properly during meiosis in hybrids. While evidence for chromosomal rearrangement leading to reproductive isolation does exist, especially in plants (e.g., Darlington 1932; see Coyne and Orr 2004; Ayala and Coluzzi 2005 for review), Dobzhansky argued that it could not be the major cause of speciation in animals. First, 4 of the 6 chromosomal inversions observed between *D. pseudoobscura* and *D. persimilis* were found on the X chromosome, and thus could not be the cause of hybrid sterility in heterogametic males. Furthermore, individuals of both sexes, heterozygous for various chromosomal inversions, were frequently observed in wild populations and showed no apparent sterility (Dobzhansky 1936).

Hermann J. Muller gave Dobzhansky's formulation a much more thorough theoretical treatment in an essay published in 1942 (Muller 1942; Orr 1996a). Firstly, he argued that incompatibilities at interacting loci rarely *began* the process of speciation, but rather *completed* it: time was required for independent divergence to accumulate between populations and migration between these populations would act to slow down this divergence. Thus pre-zygotic reproductive isolation, such as physical or behavioral barriers, generally must precede post-zygotic reproductive isolation (with occasional exceptions). Secondly he showed that incompatible interactions among loci could be 'complex' and involve more than just two-complimentary factors. Thirdly, he speculated on the biochemical basis of hybrid sterility, considering whether sterility arose as the result of loss- or gain-of-function mutations: Do incompatibilities prevent factors from activating properly, or do they lead to novel products that 'poison' the hybrid? Finally, he noted that a unique outcome of the inheritance of incompatible complimentary factors would occur if one or both of the factors were found on the sex chromosome, of which the heterozygous sex (males in the case of *Drosophila*) inherits only one, establishing the important 'Dominance Theory' of speciation genetics (see § 1.2.2).

Due to a general ignorance of the work of Bateson (1909), but more significantly due to the empirical and theoretical insights of Dobzhansky and Muller, incompatible interactions among loci leading to reduced fertility or inviability in hybrids have traditionally been called Dobzhansky-Muller (or DM) incompatibilities (Coyne and Orr 2004). With the intent of acknowledging the insight of Bateson, I shall follow the example of some recent authors (e.g., Gavrilets 2003; Palmer and Feldman 2009) and refer to the phenomenon as Bateson-Dobzhansky-Muller Incompatibilities, or BDMIs. Furthermore, given that the majority of the work performed in studying and identifying BDMIs has been performed in the genus *Drosophila*, I have provided a phylogeny composited from several recent analyses as a reference to the reader (Figure 1.2).

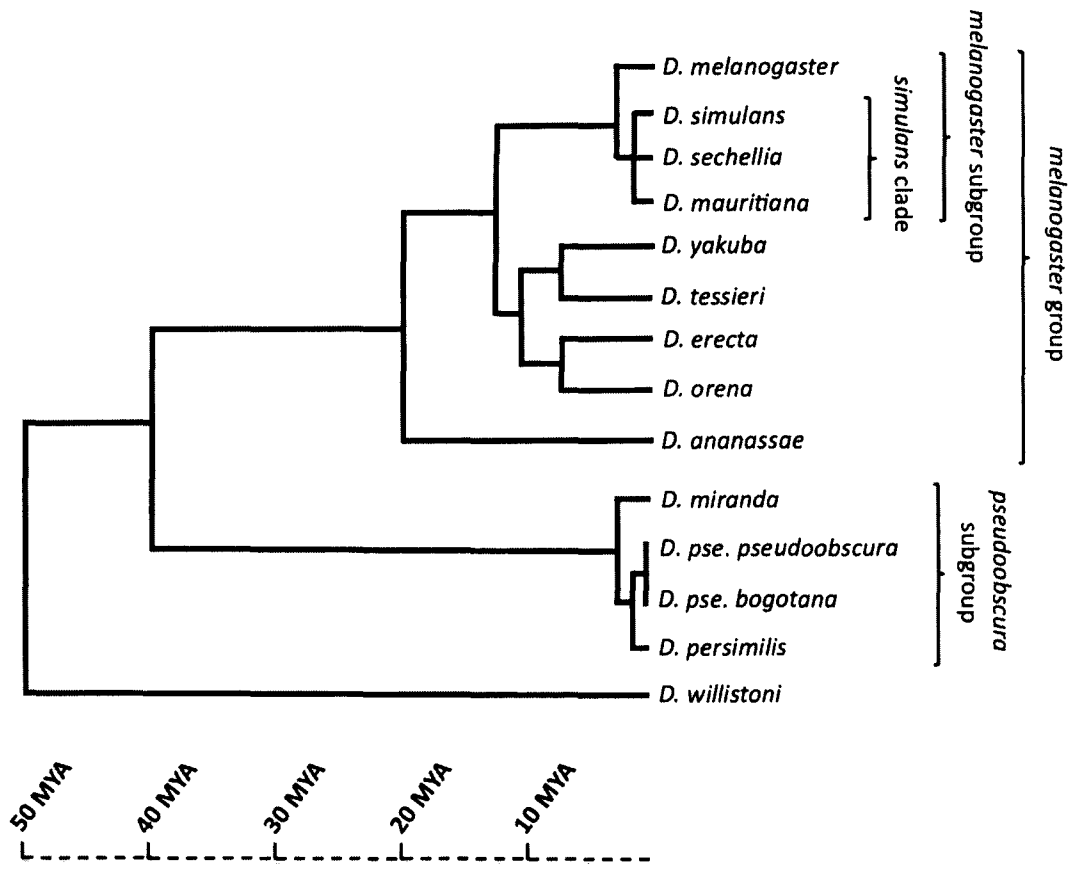

Figure 1.2: Phylogeny of the genus *Drosophila*, subgenus *Sophophora*. Species relevant to the information presented in this thesis are shown with their estimated divergence times drawn roughly to scale. In addition, the primary taxonomic divisions used by Drosophilists are also indicated. Note that '*pse.*' represents *pseudoobscura*, and 'MYA' million years ago. Both topology and divergence times were obtained by aggregating data from Powell 1997, Tamura et al. 2004, Stark et al. 2007, and Larracuente et al. 2008.

1.2.2 Haldane's rule

In a paper published in 1922, J.B.S. Haldane made an observation suggesting that the genetic factors leading to post-zygotic reproductive isolation may be very similar across a broad variety of taxa. Now known as 'Haldane's Rule', it was originally stated as follows:

> When in the F_1 offspring of two different animal races one sex is absent, rare, or sterile, that sex is the heterozygous sex. (Haldane 1922, 7)

By 'heterozygous' Haldane meant heterogametic, and he cataloged a number of examples in both XY (e.g., *Drosophila*, mammals) and ZW (e.g., Lepidoptera, birds) sex determination systems that followed this rule. It should be noted that the rule is not respected perfectly, and Haldane was aware of at least two exceptions to the universality of his postulate: an interspecific cross in the moth genus *Lymantria* observed by Goldschmidt (1920), and the cross between male *Drosophila melanogaster* and female *D. simulans* observed by Sturtevant (1920), which produces only sterile males (females die during embryogenesis [Orr 1996b]). However, he attributed such cases to unknown 'external conditions' or the unsuspected heterozygosis of one parent (Haldane 1922). While more exceptions have since been identified, subsequent studies have found that Haldane's rule is well obeyed in all taxa surveyed – in 242 out of 255 crosses according to Orr (1997; but see also Coyne 1992; Wu and Davis 1993). Furthermore, female heterogamety has evolved independently in multiple taxa (e.g., birds, Lepidoptera, and salamanders), indicating that their obeisance of Haldane's rule is directly related to the configuration of their sex chromosomes and not by accident of descent.

Any mechanistic explanation of the cause of Haldane's rule must be congruent with the Bateson-Dobzhansky-Muller model of speciation, given the strong empirical evidence that has accumulated attesting to its veracity (see § 1.2.3.1). Although many mechanisms have been proposed in order to account for the preferential sterility/inviability of heterogametic hybrids (see Kulathinal and Singh 2008 for a recent review), only three hypotheses are generally believed to be widely applicable: the dominance, faster-X, and faster-male theories (Orr 1997; though see below). The most well-supported of these mechanisms, known as the **dominance theory**, was originally proposed by Muller (1942) and subsequently refined by Orr (1993; 1995), Turelli and Orr (1995; 2000), and Orr and Turelli (1996). According to this theory, if alleles that lower hybrid fitness act as partial recessives in the hybrid, then the heterogametic sex should suffer stronger deleterious effects as, unlike in its homogametic partner, it cannot mask deleterious recessives on its sex chromosomes (note that Muller's use of 'dominance' confusingly refers to the requirement that deleterious alleles act recessively in hybrids – nothing is assumed about how the alleles act in the parental species). In the past, there has been considerable controversy about the general applicability of dominance theory (e.g., Coyne and Orr 1989), however, recent theoretical and empirical work has provided strong

support for the notion that partial recessiveness of both sterility and viability alleles in the hybrid background is the primary explanation for their being more deleterious to the heterogametic sex (Orr 1997; Coyne and Orr 2004). A study performed by Orr (1993) for example, found that in the case of two *Drosophila* species crosses in which males were inviable (*D. simulans-D. teissieri* and *D. simulans* [♂]-*D. melanogaster* [♀]), production of female hybrids homozygous for the X chromosome were also lethal, and died at the same developmental stage as the males – likely due to the unmasking of partially recessive hybrid lethals generated from X-autosome interactions. Interestingly, the same phenomenon was not observed in the case of male hybrid sterility: female hybrids of the *D. melanogaster* subgroup made homozygous for their X chromosome remained fertile, unlike their male siblings (Coyne 1985). Though originally thought surprising (see Orr 1997) this result may have been expected based upon the observation that mutations causing sterility were almost always sex-limited, while those causing inviability almost always affected both sexes equally (Lindsley and Lifschytz 1972; Ashburner 1989). Thus the discovery of male-sterility factors on the X chromosome implied nothing about the presence of female sterility factors, especially under the assumption that few loci are responsible for the initial stages of post-zygotic reproductive isolation (Turelli and Orr 1995). In another confirmation of the dominance theory's predictions, it was found that *Drosophila* species with larger non-recombining regions of their X chromosomes evolve hybrid sterility more rapidly than those with smaller regions (Turelli and Begun 1997) (this prediction does not hold in *Lepidoptera*, however [Presgraves 2002], but see below). If sterility is primarily caused by deleterious recessives being exposed in heterogametic hybrids, then species with larger non-recombining regions of their X chromsomes should present a larger evolutionary target through which incompatibilities can arise. Several subsequent studies have provided mounting empirical support that the dominance theory explains both inviability, in which the same loci generally affect males and females, *and* sterility, in which different loci affect each sex (e.g., Hollocher and Wu 1996; True et al. 1996).

Another possible mechanism explaining the disproportionate effect of hybridization on the heterogametic sex is known as the **faster-X theory**, originally proposed by Charlesworth et al. (1987). This mechanism is based on the observation that studies mapping loci involved in hybrid sterility have generally noted a disproportionate effect of the X chromosome on lowering hybrid fitness – in fact, Dobzhansky (1936) observed a large X effect in his original study demonstrating that hybrid sterility had a genetic basis (see Coyne 1992 for review). Such an effect could be explained if loci located on the X chromosome evolve more rapidly than those on the autosomes; a plausible situation if beneficial mutations are, on average, partially recessive in pure species and thus exposed directly to selection in the heterogametic sex (note that this is different from the dominance theory which requires that mutations be partially recessive *in the hybrids*). It has subsequently been shown that the faster-X theory cannot explain Haldane's rule alone, as female hybrids carry twice the number of Xs and thus should

suffer twice the effects of partially recessive mutations (see Orr 1993; Turelli and Orr 1995 for detailed mathematical formulation). Nonetheless, a faster-X effect can exaggerate the effect of the dominance theory by concentrating a larger number of speciation genes on the X chromosome in comparison to the autosomes as more rapid gene divergence is expected to accelerate the rate of accumulation of BDMIs (Orr 1997).

Support for the accelerated evolution of X-linked loci has been mixed. Several molecular studies have found evidence that X-linked genes evolve more rapidly than those found on autosomes. For instance, Thornton and Long (2002) found that paralogs that are both X-linked in *D. melanogaster* show higher rates of divergence when compared to autosomal duplicates. X-linked mammalian sperm proteins have also been found to evolve more rapidly than their autosomal counterparts (Torgerson and Singh 2003). Also, in a whole genome comparison of *D. melanogaster*/*D. pseudoobscura* orthologs, Musters et al. (2006) found a higher average rate of non-synonymous substitution per non-synonymous site, d_N, among genes on Muller element A (corresponding to the accrocentric X chromosome of *D. melanogaster* and the left arm of the metacentric X chromosome of *D. pseudoobscura*) as compared to any autosome. Conversely, Betancourt et al. (2002) compared 254 orthologs between *D. melanogaster* and *D. simulans* and found no evidence of accelerated evolution among X-linked genes. Furthermore, a study comparing the rates of evolution of genes that have always been X-linked (i.e., Muller element A) *versus* those that are only X-linked in the *D. pseudoobscura* group (i.e., Muller element D, which corresponds to the right arm of the X chromosome in this group, and chromosome 3L in the *D. melanogaster* species group), found no statistically significant increase in the rate of evolution of the former group, as may be expected (Thornton et al. 2006). Nonetheless, questions about genome-level patterns of evolution in *Drosophila* have been greatly aided by the recent release of multiple fully-sequenced and annotated genomes (Drosophila 12 Genomes Consortium 2007), and a more recent comparison of divergence patterns between the X chromosome and autosomes of *D. melanogaster*, *D. simulans*, and *D. yakuba*, all within the *D. melanogaster* group, has found not only that, on average, X-linked genes show greater evidence of adaptive evolution, but furthermore that X-linked male-biased genes (i.e., genes with a higher level of expression in males as compared to females) are among the most rapidly diverging - as would be expected if the faster-X effect is due to selection acting upon beneficial recessive mutations in heterogametic males (Baines et al. 2008).

Haldane's rule could also be explained if genes affecting heterogametic hybrids evolved more rapidly than those affecting the homogametic sex, as again, under the Bateson-Dobzhansky-Muller model, rapidly evolving proteins would be more likely to accumulate incompatibilities when interacting in hybrids (Singh 1990; Wu and Davis 1993; Wu et al. 1996). There is abundant evidence to suggest that proteins involved in male reproduction, expressed in male reproductive organs, or male-biased in their expression, evolve more rapidly than most other gene categories, presumably due to the effects of sexual selection (see Swanson and Vacquier 2002; Singh and Kulathinal 2005;

Ellegren and Parsch 2007 for review). Thus the **Faster-Male Theory** may explain the evolution of hybrid incompatibilities in species with XY sex-determination systems. On the other hand, it is difficult to imagine a similar mechanism operating exclusively in females, who in general are subject to significantly less pressure from sexual selection as compared to males (see Arnqvist and Rowe 2004; Singh and Kulathinal 2005; Hunt et al. 2009), thus a major limitation of the theory's general applicability is that it cannot explain the incidence of Haldane's rule in ZW sex determination systems (e.g., birds and *Lepidoptera*) (Orr 1997). Furthermore, given that mutations causing inviability generally involve genes that are not sex-specific (Lindsley and Lifschytz 1972; Ashburner 1989), the faster-male theory should not apply to hybrid inviability. Finally, it should also be noted that the faster-male theory could also apply to hybrid sterility in the case where male and female sterility genes evolve at similar rates if spermatogenesis is inherently more sensitive to perturbation than oogenesis (Wu and Davis 1993). In such a case while a similar number of incompatibilities could arise in both sexes, males would nevertheless be affected more severely.

Keeping in mind the limitations of its applicability, specific tests of the faster-male theory have provided strong empirical evidence of its validity (see Orr 1997; Presgraves 2008 for review). For instance, a simple prediction of the theory is that male sterility factors should arise more frequently than female sterility factors in XY species. This has been confirmed in a large scale chromosomal introgression study between *D. simulans* and *D. mauritiana* (True et al. 1996), which found that 36% of the lines featuring *D. mauritiana* segments introgressed into a *D. simulans* background caused male sterility, whereas only 7% resulted in female sterility. Similarly, another study that introgressed chromosomal segments from *D. mauritiana* into both *D. simulans* and *D. sechellia* also noted a larger proportion of male steriles as compared to female steriles (Hollocher and Wu 1996). A more recent study by Masly and Presgraves (2007) that also introgressed *D. mauritiana* genomic segments into a *D. sechellia* background found that while 33% percent of the their introgressed lines showed complete male sterility, none were female sterile. All three studies noted little or no sex-specific lethality, supporting expectations that the faster-male theory applies only to hybrid sterility. If the dominance theory explains both sterility and inviability whereas the faster-male theory explains only sterility, then we may expect that hybrid sterility will be more common than inviability in XY species, where both mechanisms play a role, in comparison to ZW systems wherein we expect only the former theory to have a significant impact. This prediction was confirmed (Wu et al. 1996), thus strongly supporting the effect of the faster-male theory in explaining reproductive isolation in XY systems (Orr 1997; Coyne and Orr 2004).

While these three explanations of Haldane's rule enjoy the most attention due to their ability to explain preferential heterogametic sterility/inviability in a wide variety of taxa, there is nonetheless a growing consensus that the underlying causes of Haldane's rule are multifactorial and that no single mechanism can explain its ubiquity (or why it fails to hold in a small number of cases, for that matter) (see Kulathinal and Singh 2008).

Nonetheless, one other factor that may play a role in explaining Haldane's observations, at least in the case of *Drosophila*, is genomic conflict involving **meiotic drive**, or the phenomenon in which selfish genetic elements distort the normal patterns of meiotic segregation in order to favor their own transmission, thus making some chromosomes appear more frequently than expected in the progeny (Tao and Hartl 2003). Under this theory, sex chromosomes may accumulate sex-ratio distorters, which will create strong selection pressure to evolve suppressors in the heterogametic sex and restore proper sex ratio balance (see Fisher 1930). Frequent cycles of distortion/suppression are expected to have the pleiotropic effect of accelerating the evolution of sex and reproduction related genes in the heterogametic sex, because these conflicts involve gametogenesis; yet unlike the faster-male theory, this rapid SRR evolution will contribute to Haldane's rule for sterility in both XY and ZW systems (Laurie 1997; Tao and Hartl 2003). Furthermore, it is possible that heterogametic hybrids inherit incompatible distorter/suppressor complexes (each having evolved independently in one lineage) and thus experience sex ratio distortion, favoring the transmission of X (or Z) containing sperm, which conforms to Haldane's rule. Alternatively, a hybrid may inherit both X- and Y-linked suppressor elements wherein the former inactivates Y bearing sperm and the latter inactivates X bearing sperm, rendering male hybrids sterile (Coyne and Orr 2004). While the scope of meiotic drive driven hybrid sterility is debated and requires further research, in the case of *Drosophila*, distortion/suppression complexes appear to be more common than was originally thought, and evidence is accruing indicating that meiotic drive may have a significant effect in explaining Haldane's rule in flies (see Coyne and Orr 2004; Orr et al. 2007; Presgraves 2007; 2008).

1.2.3 The genetics of speciation in the molecular era

A renewal of interest in the field of speciation genetics began in 1980s and benefitted from the wealth of molecular tools that had been developed in the time since Dobzhansky's initial empirical studies (Dobzhansky 1936; Orr 1997). Three developments in particular bear direct relevance to the discussion at hand: the identification of specific loci causing reproductive isolation (so-called 'speciation genes'), a greater recognition of the role played by sexual selection and sexual conflict in explaining patterns of speciation in metazoans, and finally, the recognition that BDMIs can stem from incompatibilities in the factors controlling gene regulation, such as protein-DNA/RNA interactions, in addition to traditional protein-protein interactions. I shall discuss each of these topics in turn.

1.2.3.1 Identification of 'speciation genes'

Fine-scale mapping of hybrid incompatibility factors (e.g., Tao et al. 2003a, b; Sawamura et al. 2004) has provided both confirmation of the Bateson-Dobzhansky-

Muller model, as well as a wealth of information about the genomic position of BDMI loci: revealing for example, the large effect of the X chromosome in *Drosophila*. Nonetheless, several fundamental questions about the nature of loci implicated in reproductive isolation elude our ability to provide answers so long as we remain unaware of the identity of the genes, or non-coding elements, involved (see Orr et al. 2004 for review). For instance, are there certain classes of genes (e.g., sex and reproduction related, physiological, immune system related, etc.) that are typically involved? Also, do incompatibilities generally appear due to rapid divergence driven by positive Darwinian selection, or do they arise as the by-product of neutral processes? To date, only a small number of 'speciation genes' have been identified, mostly in *Drosophila*, nevertheless they have revealed some suggestive patterns.

The first gene involved in hybrid male sterility to be identified in *Drosophila* was the X-linked *Odysseus site homeobox*, or *OdsH*, which causes sterility in hybrids of *D. simulans* and *D. mauritiana* (Coyne and Charlesworth 1986, Ting et al. 1998). When introgressed alone into *D. simulans*, the *D. mauritiana* allele of *OdsH* causes only a 50% reduction in fertility. The addition of closely linked, unidentified factors, results in total sterility, suggesting that the complete hybrid-sterility phenotype is polygenic (Perez and Wu 1995; Orr et al. 2004). The specific function of *OdsH* remains unknown; however, it is a recent duplicate of *unc-4*, a transcription factor expressed in embryonic and adult neural tissues. *OdsH,* has acquired novel expression in the testes, and knockout experiments have indicated that it is involved in sperm production, thus suggesting a biochemical link to its effect on hybrid male sterility (Sun et al. 2004). A second gene implicated in male hybrid sterility in *Drosophila* – in this case in the hybrids between *D. melanogaster* and *D. simulans*, is *JYAlpha*, a gene residing on the small 4th chromosome of *D. melanogaster* that encodes the alpha subunit of the Na^+ and K^+ adenosine triphosphatase (Masly et al. 2006). The cause of sterility in this case is unrelated to BDMIs, but rather is a by-product of the translocation of *JYAlpha* to the right arm of chromosome 3 (3R) in the *D. simulans* clade (which includes *D. sechellia* and *D. mauritiana*). Thus while F_1 male hybrids carry at least one functional copy of *JYAlpha*, a small proportion of the F_2 hybrids (those homozygous for *D. melanogaster*'s third chromosome and *D. simulans*' 4th chromosome) will lack *JYAlpha*, which is required for sperm motility (Masly et al. 2006). While this discovery raises the possibility that gene transposition may be involved in the process, it is difficult to imagine that *JYAlpha* played a substantial role in reproductive isolation, given that only 1/16 of F_2 hybrids would inherit the proper combination of chromosomes that imposed sterility and prevented gene flow. However, as Masly et al. (2006) note, Y to X transpositions of genes required for proper spermatogenesis would cause sterility in F_1 hybrids, though such a case remains to be observed in natural populations. Very recently, Phadnis and Orr (2009) discovered an X-linked gene, *Overdrive*, that causes both male BDMI-based hybrid sterility and segregation distortion (i.e., meiotic drive) in hybrids between the closely related *D. pseudoobscura pseudoobscura* and *D. pseudoobscura bogotana* (0.16 to 0.23 MYD;

Machado and Hey 2003). Given the limited amount of time that has passed since these subspecies shared a common ancestor, this raises the possibility that *Overdrive* was involved in the initial process of reproductive isolation, rather than having evolved later as a by-product of cessation of gene flow between certain populations of *D. pse. pseudoobscura* and *D. pse. bogotana* (Orr et al. 2007; Phadnis and Orr 2009).

In addition to sterility, several genes involved in hybrid inviability have also been identified in *Drosophila* (see Orr et al. 2004). The first of such genes, *Hybrid male rescue* (*Hmr*), was originally identified as a loss-of-function mutation that suppressed the normal hybrid male lethality observed in the cross between *D. melanogaster* females and *D. simulans* males (Hutter and Ashburner 1987). Cloning and subsequent identification of the locus responsible revealed that *Hmr* is a rapidly evolving, X-linked, DNA-binding protein that may have transcriptional regulation activity (Barbash et al. 2003). A second gene identified based on its ability to rescue normally inviable males in the same cross is *Lethal hybrid rescue* (*Lhr*) (Watanabe 1979; Takamura and Watanabe 1980). The *Lhr* locus was recently cloned and characterized (Brideau et al. 2006), revealing that it interacts directly with *Hmr* and confirming what was suggested by previous genetic interaction studies (Barbash et al. 2000; Orr and Irving 2000). Like *Hmr*, *Lhr* encodes a rapidly evolving, putative DNA binding domain, potentially involved in chromatin remodeling and residing on chromosome 2R. While the two loci require a hybrid genetic background in order to produce the sterility phenotype (suggesting that other genes may be involved), the interacting *Hmr/Lhr* pair represents the first example of a characterized BDMI (Brideau et al. 2006). Finally, recent work has shown that at least 2 interacting genes in the nuclear pore complex, *Nup96* and *Nup160*, are also involved in BDMIs between *D. melanogaster* and *D. simulans* (Presgraves et al. 2003; Tang and Presgraves 2009). Unlike the previous loci, the nuclear pore complex is a ubiquitous, structural feature of the cell, with no known transcriptional activity, indicating that BDMIs may not be restricted to a particular class of loci (Coyne and Orr 2004).

As indicated above, the sample size of characterized 'speciation genes' remains relatively small; however, it does allow us to infer some general patterns about the kinds of genes that are involved in post-zygotic reproductive isolation (Orr et al. 2004; 2007). Firstly, speciation loci do not appear to be the products of novel genetic processes that are directly implicated in reproductive isolation, such as the large-scale activation of transposable elements in hybrids (e.g., Rose and Doolittle 1983). Rather, these loci are 'ordinary genes' in the sense that they represent a broad diversity of functions, suggesting that no particular functional class is preferentially involved in the isolation process (though several do appear to have transcriptional activity [Noor 2005]). Secondly, many of these genes appear to be rapidly evolving, either with evidence of recent selective sweeps, or via evidence of selective forces driving sequence divergence in their past (e.g., a higher ratio of replacement to silent substitution than replacement to silent polymorphism) (McDonald and Kreitmann 1991). While the Bateson-Dobzhansky-Muller model suggests that BDMIs will accumulate regardless of selective forces if given

sufficient time, theoretical models suggest that processes driving increased divergence between species (e.g., positive selection or relaxed selective constraint) will increase the rate of accumulation of BDMIs (Orr and Turelli 1995). Thus it is perhaps unsurprising that the speciation genes that have been uncovered display evidence of positive selection, perhaps driven by adaptation to external pressures or intragenomic conflict, both of which happen to be hallmarks of the process of sexual selection (Orr 2007; Kulathinal and Singh 2008).

1.2.3.2 Sexual selection and speciation

The formal proposal of an association between sexual selection and speciation dates back at least to Fisher's *The Genetical Theory of Natural Selection* (1930), wherein, after proposing his well-known 'sexy sons' hypothesis explaining how female preference for male characters can evolve rapidly, he noted that:

> An important means of fission [speciation], particularly applicable to higher animals, lies in the possibility of differential sexual response [female preference] to differently characterized suitors. Circumstances favourable to the fission of species into parts adapted to different habitats will also be favourable to the development both of discrimination and sexual preference. (Fisher 1930, 144)

Thus he argued that sexual selection could accelerate the process of speciation by creating pressure for rapid divergence in male traits as well as female preference between populations. Lande (1981), formalized Fisher's sexy sons hypothesis mathematically under the assumption that that both female preference and male traits were polygenic (previous models had employed only two loci [e.g., O'Donald 1980]). Under such conditions, he was able to show that 1) sexual selection caused male traits to diverge almost exponentially in rate until an equilibrium was reached between sexual selection driving trait elaboration and natural selection against the maladaptive property of the exaggerated trait, and that 2) the diversity of possible male traits and equilibrium positions was enormous; thus implying that it was extremely unlikely that two populations would settle upon the same equilibrium. As Lande noted, "these models help explain the classical observations of Darwin... that closely related species differ most in the characters of adult males" (1981, 3725). Though these inferences were concerned with the establishment of pre-zygotic reproductive isolation (e.g., different female preferences and male traits between populations), and have been confirmed experimentally in the life-long work of H.L. Carson (see Carson 1997; 1999 for review), more recent theory has sought to also link sexual selection directly to post-zygotic reproductive isolation.

While pre- and post-zygotic isolation mechanisms may have very different ecological implications, their association with sexual selection may derive from the same

mechanistic basis: accelerated divergence of loci between populations. Drawing on data compiled from a variety of authors, Singh (1990) argued that sex and reproduction related genes, in addition to secondary sexual characteristics, evolve unusually rapidly between species (see Civetta and Singh 1995, 1998; Swanson and Vacquier 2002 for recent treatments). Furthermore, evidence indicated that interspecific hybrid sterility evolved faster than inviability in *Drosophila* species (Bock 1984), suggesting that factors involving reproduction played a key role in the speciation process. Thus Singh proposed a 'reproductive model of speciation', suggesting that the process of reproductive isolation preferentially involved sex and reproduction related genes given that their rapid interspecific divergence predisposed them to being involved in BDMIs (Singh 1990). This hypothesis has been subsequently confirmed via numerous studies, and evidence suggests that sexual selection driven rapid evolution of reproductive proteins is largely responsible for the 'faster-male' effect (see § 1.2.2; reviewed in Coyne and Orr 2004; Kulathinal and Singh 2008). Therefore, it has traditionally been argued that the association between sexual selection and speciation has the same limited applicability as the faster-male theory in general: it only applies to male heterogametic species and can only explain hybrid sterility, not inviability. However, Kulathinal and Singh (2008) have recently proposed a 'hierarchical faster-sex theory of speciation' that seeks to implicate the rapid evolution of reproductive proteins in the evolution of sterility in ZW taxa as well. This theory rests on various lines of evidence indicating that not only male, but rather all sex and reproduction-related genes undergo some degree of rapid evolution - driven not only by sexual selection acting more strongly on males, but also by such processes as sexual conflict, which applies equally to female heterogamety (see Arnqvist and Rowe 2004 for review). Thus while XY systems may experience a more severe bias towards the preferential evolution of hybrid sterility over inviability due to the action of sexual selection (see Coyne and Orr 2004; but also Presgraves 2002), ZW species are nonetheless experiencing an elevated rate of evolution of reproductive proteins that may likely contribute to the accumulation of BDMIs (e.g., Mank et al. 2007; Kulathinal and Singh 2008). Though the extension of sexual selection/conflict theory to ZW taxa has the potential to improve our understanding of Haldane's rule, empirical confirmation of Kulathinal and Singh's (2008) proposal will have to await the collection of more molecular data in the heterogametic sex of ZW taxa - or females in general, for that matter, as the overwhelming majority of speciation studies have looked exclusively at males (Coyne and Orr 2004).

1.2.3.3 Studies of speciation at the level of gene expression

Since the general acceptance of the Bateson-Dobzhansky-Muller model of hybrid incompatibility, it has been recognized that a central feature of the genetics of post-zygotic reproductive isolation is their dependence on epistasis – or non-additive genetic interaction among loci (reviewed in Coyne and Orr 2004). Interestingly, despite

this emphasis on interaction, the possible association between hybrid sterility/inviability and gene regulation – a process mechanistically characterized by complex and specific interactions between proteins and proteins and/or nucleic acids (e.g., Morisato and Anderson 1995) – has, until recently, remained largely unexplored (see Ortíz-Barrientos et al. 2007 for review). As Johnson and Porter (2000, 528) have emphasized "...regulated genetic pathways are a biologically realistic way to provide the complex epistatic gene interaction seen in empirical studies of hybrid fitness reduction." The first forays into the study of transcriptional dynamics as a mechanism of hybrid sterility/inviability were theoretical, modeling how interspecific divergence of transcription factors (TFs) and regulatory binding sequences (*cis* elements) involved in complex pathways, would manifest themselves in hybrid genomes (Johnson and Porter 2000, 2001; Porter and Johnson 2002). Several interesting conclusions were drawn from these models. Firstly, it was found that hybrids could experience reduced fitness in situations where selection had driven divergence of TF/binding site affinities. Interestingly, this was predicted to occur even under conditions in which both lineages experienced similar selective pressures, due to complex regulatory pathways containing many possible ways of meeting the demands of selection on a given phenotype (Johnson and Porter 2000). As a consequence, models also predicted that the likelihood of incompatibilities arising was directly proportional to the underlying complexity of binding site interactions. Finally, an interesting, if somewhat paradoxical, prediction made by these models was that genes showing high intraspecific variability in gene expression were less likely to be involved in hybrid incompatibilities. This follows from the notion that highly variable genes are more likely to tolerate a greater range of transcriptional binding affinities, thus making the hybrids less likely to be adversely affected by improper binding (see Ortíz-Barrientos et al. 2007 for discussion).

These models were rapidly followed by experimental comparisons of the expression profiles of interspecific hybrids to their parents. Reiland and Noor (2002) analyzed the expression patterns of one day old adult males of the pure species and F_1 hybrids of *D. persimilis* and *D. pseudoobscura* using differential display (Liang and Pardee 1992; 1997) – a technique that both lacks sensitivity as compared to microarrays or quantitative PCR (qPCR) and confounds sequence and expression differences between the species surveyed. Consequently, they confirmed only a single gene that was differentially expressed in the F_1 hybrids as compared to either of the parents. However, their data suggested that hybrid expression profiles were quantitatively different (i.e., expression levels) from those of the parents rather than qualitatively different (i.e., overall expression patterns) (Reiland and Noor 2002). Further investigations of hybrid expression switched to oligonucleotide (Michalak and Noor 2003, Barbash and Lorrigan 2007) and cDNA (Ranz et al. 2004; Haerty and Singh 2006; Moehring et al. 2007) microarrays, or qPCR (Michalak and Noor 2004), from which several significant patterns emerged (reviewed in Ortíz-Barrientos et al. 2007). Firstly, confirming the results obtained by differential display, patterns of gene misexpression in *Drosophila* species hybrids - where

misexpression is defined as genes having an expression level that is significantly different from both parents and not at an intermediate level between parents (Michalak and Noor 2003) – tend to involve quantitative changes. This typically manifests itself as a number of genes being underexpressed relative to the parents; though overexpression is also observed at a lower frequency. Secondly, MBGs (specifically those expressed in the testes) are consistently overrepresented among genes showing significant hybrid misexpression, suggesting that similar factors are implicating these genes in hybrid dysfunction at the nucleotide and expression levels (i.e., rapid evolution due to sexual selection or relaxed selective constraint). Thirdly, the proportion of the transcriptome that shows significant misexpression in hybrids is positively correlated with divergence time: species whose common ancestors occurred less recently tend to have a greater proportion of their total mRNA misexpressed. A word of caution is required here: hybrids generated by crossing distantly related species tend to show more morphological defects than do hybrids derived from more closely related pairs (Coyne and Orr 2004). In particular, both male and female hybrids of *D. melanogaster* and *D. simulans*, as used in the studies of Ranz et al. (2004) and Haerty and Singh (2006), display severe gonadal atrophy (Sturtevant 1920), thus confounding differential gene expression and allometric effects engendered by differing tissue abundance (Ortíz-Barrientos et al. 2007). Finally, comparisons of the expression profiles of hybrids generated from multiple, closely related species, such as those of the *D. simulans* clade, showed that very few genes are commonly misexpressed in different hybrids, suggesting that the genetic architecture of hybrid sterility involves the lineage-specific accumulation of BDMIs in different genetic systems, rather than incompatibilities consistently arising in common systems (Haerty and Singh 2006).

A few specific observations drawn from hybrid expression studies warrant more detailed discussion. The direction of causality with regards to hybrid misexpression, like any phenotype, remains unclear: Is misexpression responsible for hybrid sterility (or inviability) or are other incompatibilities producing sterility, which then leads to a misexpression phenotype? Michalak and Noor (2004) used qPCR to survey the expression levels of 5 genes shown to be misexpressed in the male F_1 hybrids between *D. simulans* and *D. mauritiana* in 5th generation backcross hybrids. Both fertile and sterile males are represented among the 5th generation backcross males, and the latter display significantly reduced expression levels of these transcripts as compared to the former. Interestingly, the defects causing sterility in *D. simulans* (♀) × *D. mauritiana* (♂) hybrids are post-meiotic, indicating that they occur *after* spermatogenic chromosome condensation and cessation of transcriptions occurs. Thus spermatogenic arrest cannot be the cause of misexpression. While this does not causally associate misexpression with sterility conclusively, it does suggest that improper expression is not simply a by-product of the mechanism of sterility (Michalak and Noor 2004). The situation pertaining to hybrid inviability is less clear. The male hybrids generated from the cross between female *D. melanogaster* and male *D. simulans* die as larvae or undifferentiated pupae (Sturtevant

1920). Barbash and Lorrigan (2007) examined the patterns of gene expression in male *Hmr*$^{+}$ (lethal) and *Hmr*$^{-}$ (rescued) hybrids at the late 2nd instar larval stage, and found that between 70 and ~190 genes were differentially expressed between lethal and rescued hybrids (depending on the analysis methodology employed). From this observation, they concluded that lethality is not associated with substantial transcriptional misregulation, though they could not rule out the possibility that the differential expression was, in fact, causally associated with lethality (Barbash and Lorrigan 2007).

Also of note is the study of Ranz et al. (2004), which examined the whole-body extracted mRNA cDNA microarray expression profiles of the sterile, **female** hybrids of the cross between *D. melanogaster* (♀) and *D. simulans* (♂) – one of the rare studies involving a homogametic hybrid. The authors found that ~69% of the genes represented on their microarray platform were misexpressed in the hybrids relative to their female parents. However, as Ortíz-Barrientos et al. (2007, 76) remark, this number is 'strikingly high' and likely reflects a combination of morphological defects and sequence divergence confounding legitimate expression differences (see above). Nonetheless, the study noted that, female-biased genes are over-represented among those underexpressed in hybrids. Furthermore, they noted that MBGs are over-represented among those overexpressed in the female hybrids, suggesting that while a loss-of-function like phenotype leading to underexpression of transcripts in hybrids is the most common phenotype, there also appears to be some loss of the ability to properly repress genes that should not be expressed at high levels (i.e., MBGs in females).

Despite the wealth of suggestive patterns that have been derived from the study of gene expression in interspecific hybrids, several authors have urged an important note of caution in the interpretation of such data. With the exception of a single study that compared the expression patterns of 48 testis-specific genes in larvae to those of the adults (Moehring et al. 2007), all *Drosophila* hybrid expression studies have been performed in single developmental stages. Years of developmental genetics work has indicated that the regulation of gene expression occurs via the action of complex regulatory cascades, in which the products of one step in a regulatory hierarchy determine the expression of the products in subsequent, and even previous steps via feedback loops, (see Wright 1990; § 1.3.2). Therefore, it is possible that the number of genes observed to be misexpressed in adult hybrids does not reflect widespread, incompatible divergence of regulatory factors at this stage, but rather the downstream, cascading effect of a smaller number of factors that are misexpressed in earlier stages of development (Reiland and Noor 2002; Ortíz-Barrientos et al. 2007). Consequently, studies of hybrid gene expression at a number of different developmental stages will be required to address this possibility.

1.3 Ontogeny and Phylogeny

> "Let's be honest: the much hyped discipline of evolutionary developmental biology, or evo-devo, hasn't quite lived up to expectations, at least not if we're expecting a revolution in biology... [F]or the most part, evolutionary biology has been enriched by evo-devo, whereas developmental biology has not."
>
> -Michael Richardson, A naturalist's evo-devo (2003)

1.3.1 Development in the context of evolution

In spite of Darwin's reference to the science of 'development and embryology' as "... one of the most important subjects in the whole round of history," (Darwin 1872, 586), as well as his having claimed that:

> Hardly any point gave me so much satisfaction when I was at work on the Origin, as the explanation of the wide difference in many classes between the embryo and the adult animal, and of the close resemblance of the embryos within the same class. (Darwin 1887, 88)

the fields of developmental and evolutionary biology saw very little overlap for the better part of the 20th century (reviewed extensively in Gilbert et al. 1996; Raff 1996). As both Gould (1977) and Raff (1996) have surmised, this lack of proper integration stems from the dissatisfaction felt by the rising, empirical German school of *entwicklungsmechanik*, or developmental mechanics, with the fancifully descriptive but ultimately false Haeckelian theory of recapitulation, captured in Haeckel's famous 'biogenic law': "ontogeny recapitulates phylogeny" (Haeckel 1866). Recapitulatory theory did not survive its collision with the rediscovery of Mendelian genetics, and unfortunately, neither did the nascent attempt to properly unite development (ontogeny) and evolution (phylogeny) (Gould 1977). It was not until the 1970s that a major reinvigoration of interest in the evolution of development occurred, fueled by at least two circumstances. Firstly, it was suggested that closely related species such as chimpanzees and humans were too similar at the nucleotide sequence level to explain the morphological disparity between them, implicating changes at the level of gene regulation in order to account for their morphological divergence (King and Wilson 1975). Secondly, the genetic mechanisms regulating segmentation in *Drosophila* were discovered, representing an early success story that established the field of developmental genetics (Lewis 1978; Nusslelein-Volhard and Weischaus 1980; see Johnson and Porter 2001 for review). These genes, called HOX proteins because they share a common DNA binding domain known

as the homeodomain box, were soon also found to be present in frogs, mice, humans, birds, and fish (McGinnis et al. 1984, see Raff 1996). Furthermore, not only were the sequences of HOX genes remarkably conserved (transgenic insertion of the human enhancer region of homeotic genes can restore function of *Drosophila* null mutants [McGinnis et al. 1990; Malicki et al. 1992]), but also their collinear chromosomal arrangement is also largely conserved among metazoans (McGinnis and Krumlauf 1992; Krumlauf 1992; Bachiller et al. 1994). The discovery that common regulatory mechanisms could underlie the developmental program of all animals, coupled to the emergence of molecular technologies required to study these mechanisms, led to the establishment of the field of evolutionary developmental biology (evo-devo).

Using the tools of developmental genetics, evo-devo has enjoyed a large number of successes in elucidating fundamentally similar developmental pathways across very divergent organisms. These include the discovery of *Pax-6*, a gene regulating eye development in both *Drosophila* and humans (Quiring et al. 1994), the *Xenopus* gene *chordin* that regulates gastrulation in both frogs and flies (François and Bier 1995; Holley et al. 1995), and *Csx/tinman*, which controls the development of the heart in both vertebrates and flies (Manak and Scott 1994), among others (reviewed in Gilbert et al. 1996). Such 'master regulators' have forced us to reconsider our notions of homology and analogy: the eyes of flies and humans have often been touted as an example of the latter, however, they seem ultimately to stem from an ancestrally derived mechanism - termed 'homologies of process' in the language of Gilbert et al. (1996). Unfortunately, as indicated above (§ 1.1), despite these successes, a chasm continues to separate contemporary evo-devo and mainstream evolutionary biology, with the latter dominated by its emphasis on population genetics and the fundamental assumption that macroevolutionary patterns may be extrapolated from the study of microevolutionary processes (Johnson and Porter 2001). Evo-devo, for the most part, focuses on a completely different scale of evolutionary history, and places an emphasis on describing highly conserved processes among widely divergent taxa. Richardson (2003, 351) summarized the general discord between research interests of evolutionary biologists and enthusiasts of evo-devo in a single statement: "The problem with 'universals' is that they make for bad phylogenetics."

Unfortunately, until very recently, the effort to unite development with evolution as envisioned by Gould (1977) has come almost entirely from the side of developmental biologists. With a view to discuss the progress made in rebalancing 'evo-devo' from what has largely been 'devo-evo', I shall sketch a brief history of the relationship between ontogeny and phylogeny with regards to its earliest and most significant overall phylogenetic trend from the point of view of population genetics: the greater conservation of features of early development among species. I shall ignore any discussion of Haeckel's theory of recapitulation as it has been thoroughly reviewed and discredited in previous literature (De Beer 1940; Stebbins 1950; Gould 1977; Raff 1996). Furthermore, I shall use the terms 'development' and 'ontogeny' interchangeably, after the fashion of

Gould (1977), though this need not be the case, however, as development is typically concerned with the period between fertilization and adulthood, while ontogeny can be taken to span the entire interval between fertilization and eventual age-related death. Given that I am primarily concerned with the former interval (fertilization to adulthood), I shall also ignore evolutionary hypotheses pertaining specifically to adult ageing and senescence (e.g., Medawar 1952, Williams 1957).

1.3.2 Von Baer's 'laws'

The origins of a rigorous science of comparative embryology can be traced to Karl Ernst Von Baer, who in 1828 published his *magnum opus* of descriptive anatomy, *Entwicklungsgeschichte der Tiere*, or 'The History of Animal Development' (Von Baer 1828). During the course of his attack on the then fashionable pre-Darwinian theory of recapitulation (see Gould 1977 for review), Von Baer enumerated four observations, or 'laws' relating the course of development within species to patterns of similarity between species (I follow Gould's [1977, 56] translation of these laws):

1. The general features of a large group of animals appear earlier in the embryo than the special features.

2. Less general characters are developed from the most general, and so forth, until finally the most specialized appear.

3. Each embryo of a given species, instead of passing through the stages of other animals, departs more and more from them.

4. Fundamentally therefore, the embryo of a higher animal is never like [the adult of] a lower animal, but only like its embryo.

All four laws may be summarized in the following statement: species tend to resemble one another at the morphological level to a greater degree in earlier developmental stages than in later developmental stages, and species-specific characteristics develop from more general features that are found in other, similar species. While developmental biology has focused on explaining the complex mechanisms underlying ontogeny within species, a major interest of researchers seeking to study development in an evolutionary context has been to explain Von Baer's laws (see Raff 1996 for review).

Perhaps unsurprising given the quotations above (§ 1.3.1), the first attempt to provide an evolutionary explanation of Von Baer's laws was made by Darwin himself. In *On the Origin of Species* he first noted that the specialized characteristics of species in their adult stage rarely appeared on their offspring before they were useful, suggesting that selection generally only operated on traits expressed during the developmental stage

in which they were useful (Darwin 1859, 576-580). He then provided an explanation for why the adult stages of closely related species would be more diverged at the morphological level than earlier stages:

> Whatever influence long-continued exercise or use on the one hand, and disuse on the other, may have in modifying an organ, such influence will mainly affect the mature animal, which has come to its full powers of activity and has to gain its own living; and the effects thus produced will be inherited at a corresponding mature age. Whereas the young will remain unmodified, or be modified to a lesser degree, by the effects of use and disuse. (Darwin 1859, 578)

Thus Darwin argued that organisms show greater difference in the morphology of their adult stage because there is *greater opportunity for selection to act upon them at this stage* due to the 'varying conditions of existence' experienced later in life. The embryos of closely related species are exposed to very similar environments (e.g., the egg or placenta) when compared to adults, whose greater ability of locomotion allows them to encounter diverse environments and challenges, and therefore greater opportunity for selection. Darwin then expanded upon his original hypothesis in his later publication, *The Descent of Man*, wherein he drew upon his extensive catalogue of secondary sexual characteristics as an example of traits that were only of use to their possessors in the adult stage, and were among the most divergent traits between closely related species (Darwin 1882, 779-786). Given that he argued that the divergence of these traits was effected by sexual selection, which presumably occurs only during the adult, reproductive stage, they provided evidence that increased opportunity for selection explained the greater divergence of adult characters as compared to those of earlier stages. Somewhat curiously, Darwin's hypothesis, which we have termed the **'selection opportunity hypothesis'** (see Chapter 4), has received very little treatment in more recent evo-devo literature. For instance, in his *Ontogeny and Phylogeny*, a work that may have largely contributed to reigniting an interest in matters of development among evolutionary biologists, Gould presents the hypothesis only as an endnote, and not in the context of any major discussion of Darwin's interpretation of Von Baer's observations (Gould 1977, 419 no. 29). Rather, another hypothesis, in this case based on the action of purifying selection, has received the lion's share of current attention.

Pervasive in the modern developmental literature is the notion that mutations in genes acting at early stages of development are more likely to upset the entire downstream developmental sequence, due to a high level of pleiotropy, as compared to evolutionary divergence at later stages. Thus purifying selection is expected to act more strongly upon traits and genes expressed at early stages, constraining their divergence (see Raff 1994, 1996 for extensive review). This hypothesis has been referred to by several different names in the literature, e.g. 'developmental burden' by Reidl (1978), or 'the pleiotropy model' by Cutter and Ward (2005), etc., often leading to some confusion.

In order to simplify matters I shall place under the umbrella of the '**developmental constraint hypothesis**' all notions that suggest that because traits expressed early in development specify the action of downstream targets, they should be under stronger purifying selection than traits expressed later due to the potentially deleterious cascading effects of mutation - be they at the level of regulation, genes, or morphology (see Raff 1996, 292-320 for extensive discussion of different methods through which developmental constraint may be manifested).

The formalization of the developmental constraint hypothesis is typically credited to Reidl (1978); however, it is stated explicitly in the context of plant genetics in Stebbins (1974):

> In many instances, embryonic or seedling stages of specialized plants resemble corresponding stages of unspecialized, or primitive forms more closely than the adults of the forms in question resemble one another. This phenomenon is based upon the general principle that genes which affect later stages of development are less likely to upset the entire sequence than those which act at early stages. Hence late-acting genes, if they confer an equal adaptive advantage with respect to some particular characteristic, have a greater chance of becoming established in the population by the action of natural selection than have genes that act early in development. (Stebbins 1974, 113)

Nonetheless, Riedl's (1978, [80, 105-128]) treatment is rightfully recognized as being much more thorough. In his discussion, Riedl defined the concept of 'burden' as the responsibility (or ultimate effect) carried by a process. Furthermore:

> The degree of burden is genetically specified by the number of subsequent decisions that depend on the preliminary decision or by the number of single events (or features) functionally dependent on a preliminary decision or on a fundamental event (or feature). The functional or hierarchical position of a feature therefore plays a large role. (Riedl 1978, 104)

By distinguishing between 'events' and 'features' Riedl indicated that his hypothesis applied both at the level of genetic hierarchies (e.g., Wright 1990) and morphology. However, as Raff (1994, 1996) has noted, the developmental constraint model cannot possibly be applied monotonically over the entire course of ontogeny: Von Baer's observations apply to the majority of embryonic development, yet they do not always apply to the *earliest* periods of development, i.e., the stages immediately following fertilization. For instance, as has been known for a considerable length of time, the period of embryogenesis during which all vertebrates most resemble one another (known as the 'phylotypic' stage) is not the earliest stage of development, but rather occurs during the pharyngula stage, after neurulation has begun (Seidle 1960; Sander 1983; Galis and Metz

2001; but see also Figure 1 in Richardson et al. 1998 for visual comparison among vertebrate embryos). In fact, many examples of rapid divergence between the earliest stages of ontogeny have been documented (e.g. Raff and Kaufman 1983; Elinson 1987), indicating that hypotheses seeking to explain Von Baer's laws require a greater integration with the fundamental regulatory mechanisms controlling embryogenesis.

In order to reconcile the developmental constraint hypothesis with what was known about the variation observed in early development, Raff (1996) proposed what is known as the '**developmental hourglass hypothesis**' - a more thorough elaboration of a similar notion suggested by Seidle (1960). The developmental hourglass hypothesis differs from Stebbins' (1974) and Riedl's (1978) concept of constraint in that it does not simply assume that 'burden' decreases linearly over time: rather Raff argues that burden, and thus constraint on divergence, will be highest during those stages wherein regulatory systems are involved in the greatest number of functional interconnections or interactions. Thus, the earliest developmental stages consist of global axial patterning involving relatively few regulatory modules, which may allow considerable evolutionary flexibility (Raff 1994). However, the mid-stage of embryonic development, represented by the initiation of organogenesis, is dominated by inductive interactions among organ primordia during which any small mutational change is likely to lead to pleiotropic effects throughout the developing embryo (Galis and Metz 2001). Finally, later stages of development, while complex, are highly modularized by division into separate organs, thus increasing the likelihood that mutations will affect only specific features of development. Under Raff's model, purifying selection, and thus constraint on divergence, should be highest during those stages showing the least amount of modularity (or conversely the greatest number of interactions), irrespective of when those stages occur in the overall process of ontogeny (Raff 1994, 1996).

Perhaps unsurprisingly given its lack of treatment within the literature, it is unlikely that any published studies have attempted to test Darwin's selection opportunity hypothesis directly (though see Cutter and Ward 2005; Davis et al. 2005 for implicit discussions). On the other hand, the developmental constraint hypothesis, as well as Raff's modified development hourglass, have enjoyed considerable empirical support (e.g., Galis and Metz 2001). For more details on previous studies examining both of these hypotheses see chapters 4 and 5.

1.4 Aims of the Thesis

A complete, functional description of the factors influencing evolutionary divergence at either the genomic or phenotypic levels cannot ignore development. Von Baer's laws suggest that the relative developmental timing of gene and/or regulatory element action may have an effect on constraining divergence either by suppressing new mutations (developmental constraint) or limiting opportunity for natural selection to act

(selection opportunity). Elucidating the genetic and regulatory mechanisms underlying the greater degree of phenotypic similarity seen in early developmental stages is also important in understanding how genotypes ultimately produce phenotypes, which has been considered a 'black-box' within the field of population genetics (Johnson and Porter 2001; but see Rice 1998, 2000; Hansen and Wagner 2001; Wolf et al. 2001 for examples of population genetics models that have attempted to incorporate development and gene regulation). Models of the divergence of ontogeny based on developmental constraint and selection opportunity, or both – these hypotheses are not mutually exclusive - make different predictions about how loci diverge in a developmental context. Consequently, understanding how developmental function affects divergence is a crucial step in a modern synthesis of population genetics and development.

The overall goal of the material presented herein has been to study patterns of divergence between species with a special focus on development. As indicated in the previous discussion, most studies in the field of speciation have focused on adult phenotypes, which are obviously the end-result of developmental processes. Given the fundamental role of epistasis underlying the genetics of post-zygotic reproductive isolation, I have chosen to study how the regulation of gene expression, a process characterized by its emphasis on protein-protein and protein-DNA interactions, diverges between species as well as how this divergence is manifested in interspecific hybrids. In addition, gene expression acts as crucial (yet ill understood) intermediate between genotype and ultimate phenotype, and thus may help elucidate the relationship between the two. Finally, as will be established in the following chapters, sexual selection plays a prominent role in both speciation and development and thus has acted as a recurrent, unifying principle throughout my research.

Chapter 2 describes a genome-wide comparative study of the rates of evolution of sex and non-sex genes in three species of the genus *Caenorhabditis*. This genus contains both dioecious (having separate males and females) and androdioecious (having males and self-fertile hermaphrodites) species, the latter of which are expected to be subject to weaker sexual selection pressure. Thus we were able to test whether greater sexual selection pressure leads to a more rapid rate of evolution in sex and reproduction related genes – something frequently inferred, but not explicitly tested at a genomic level. Furthermore, we also used our whole genome data in order to estimate the rates of deleterious mutation per genome per generation in both mating types, allowing us to test the hypothesis that dioecy is maintained due to its superior efficiency at purging deleterious mutations.

Chapter 3 presents a study that examined the relationship between gene expression level and nucleotide sequence divergence, both in the context of how these diverge between species as well as how they affect gene misexpression in interspecific hybrids of *Drosophila melanogaster* and *D. simulans*. Models of how divergence of gene regulatory elements may be involved in the process of post-zygotic reproductive isolation

are often based upon the notion that expression level is subject to similar selective pressures as is the coding sequence of genes (e.g., sexual selection). Thus we tested for a positive correlation between measures of coding sequence and expression divergence, as well as whether genes with high levels of the former tend to be more significantly misexpressed in interspecific hybrids: a reasonable assumption under the Bateson-Dobzhansky-Muller model wherein rapidly diverging genes are more likely to be involved in incompatibilities.

Chapter 4 involves a study that sought to test whether genes expressed in early stages of development are more likely be conserved between species of *Drosophila*, as would be assumed from Von Baer's morphological observations (§ 1.3.2). Using data derived from stage-specific EST libraries, we sought to test whether the developmental constraint or selection opportunity models provided a better explanation for this developmental conservation by examining if genes expressed at earlier stages were involved in a larger number of protein-protein and genetic interactions (as predicted by the former hypothesis) and whether the proportion of genes showing significant evidence of positive selection increased over the course of development (as would be predicted by the latter hypothesis). Furthermore, using gonad-specific EST libraries derived both from adult and embryonic tissues, we were able to test Darwin's hypothesis that selection generally only affects genes expressed at the stage during which selection pressure occurs by observing whether embryonic gonad genes evolve rapidly such as is observed in adult gonads (presumably due to sexual selection pressure).

Chapter 5 presents a study conducted with the intent of observing how patterns of gene expression and gene regulation are conserved among males of closely related species of the *Drosophila melanogaster* group (*D. melanogaster*, *D. sechellia*, and *D. simulans*), over several stages of ontogeny (3rd instar larval, early pupal, late pupal, and adult). In addition to observing patterns of expression in pure species, via the use of *D. melanogaster* cDNA microarrays representing ~10,000 unique genes, we also profiled expression levels in the same stages of *D. simulans* (♀) × *D. sechellia* (♂) F1 male hybrids in order to determine whether the widespread patterns of misexpression previously observed in adults of these species results from the downstream cascading effect of a smaller number of genes misexpressed earlier in development, or whether regulatory incompatibility occurs in a more stage-specific, autonomous manner.

Finally, chapter 6 involves a brief conclusion, including a discussion of the overall significance of the work presented in this volume, with special attention to its implications in terms of uniting evo-devo with the field of population genetics.

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CHAPTER 2

Sexual Selection and Maintenance of Sex: Evidence from Comparisons of Rates of Genomic Accumulation of Mutations and Divergence of Sex-Related Genes in Sexual and Hermaphroditic Species of *Caenorhabditis*

This chapter has been published in Molecular Biology and Evolution:

Artieri Carlo G., Wilfried Haerty, Bhagwati P. Gupta, and Rama S. Singh. 2008. Sexual selection and maintenance of sex: Evidence from comparisons of rates of genomic accumulation of mutations and divergence of sex-related genes in sexual and hermaphroditic species of Caenorhabditis. *Molecular Biology and Evolution* 25, no. 5 (May): 972-979.

In this article, we took advantage of three fully sequenced nematode genomes (*C. elegans* and *C. briggsae*, both androdioecious, and the dioecious *C. remanei*) in order to test the hypothesis that greater opportunity for sexual selection, as expected in dioecy, leads to more rapid evolution of reproductive proteins. We also used our genomic dataset to supplement previous attempts that sought to determine whether dioecious species suffered a higher deleterious mutation rate (U) than androdioecious species. W. Haerty and I conceived of the study and were assisted by B.P. Gupta in obtaining and preparing the genomic datasets. I performed the majority of the data analysis with assistance from W. Haerty with regards to statistical inferences. Finally, I drafted the manuscript and edited it with the assistance of all authors. Please note that supplementary materials can be found online at http://mbe.oxfordjournals.org/.

2.1 Abstract

Several hypotheses have been proposed to explain the persistence of dioecy despite the reproductive advantages conferred to hermaphrodites, including greater efficiency at purging deleterious mutations in the former. Dioecy can benefit from both mutation purging and accelerated evolution by bringing together beneficial mutations in the same individual via recombination and shuffling of genotypes. In addition, mathematical treatment has shown that sexual selection is also capable of mitigating the cost of maintaining separate sexes by increasing the overall fitness of sexual populations, and genomic comparisons have shown that sexual selection can lead to accelerated evolution. Here we examine the advantages of dioecy vs. hermaphroditism by comparing the rate of evolution in sex related genes and the rate of accumulation of deleterious mutations using a large number of orthologs (11, 493) in the dioecious *C. remanei* and the hermaphroditic *C. briggsae.* We have used this dataset to estimate the deleterious mutation rate per generation, *U*, in both species and find that while it is significantly higher in hermaphrodites, both species are at least two orders of magnitude lower than the value required to explain the persistence of sex by efficiency at purging deleterious mutations alone. We also find that genes expressed in sperm are evolving rapidly in both species; however, they show a greater increase in their rate of evolution relative to genes expressed in other tissues in *C. remanei*, suggesting stronger sexual selection pressure acting on these genes in dioecious species. Interestingly, the persistence of a signal of rapid evolution of sperm genes in *C. briggsae* suggests a recent evolutionary origin of hermaphrodism in this lineage. Our results provide empirical evidence of increased sexual selection pressure in dioecious animals, supporting the possibility that sexual selection may play an important role in the maintenance of sexual reproduction.

2.2 Introduction

Males and females of dioecious species (species with independent sexes) often display highly dimorphic secondary sexual traits (Eberhardt 1985). Darwin proposed the theory of sexual selection (Darwin 1871) in order to explain why traits that increase the reproductive success of individuals (irrespective of their survival) may be favored and maintained. Mathematical treatments of sexual selection in dioecious species have shown that the strength of selection is dependent on the amount of effort that is invested in searching out and acquiring mates (usually by males) versus taking part in other parental activities such as maturing eggs or rearing offspring (Sutherland 1985, 1987). Under such theory, self-fertile hermaphroditic species are believed to be subject to weaker pressures of sexual selection, due to reduced competition for mates (Greeff and Michiels 1999). The same will also be true of androdioecious species (i.e. species with self-fertile hermaphrodites in addition to separate males) albeit with expectations of a less extreme effect. Recent empirical observations support such theoretical predictions. For example,

Chasnov et al. (2007) found that hermaphrodites of the species *Caenorhabditis elegans* and *C. briggsae* have lost the ability to produce male-attracting pheromones, presumably because reproduction in these species occurs primarily by hermaphroditic self-fertilization and there is little selection pressure to retain the ability to attract males.

The mainstream hypothesis explaining the maintenance of dioecy is the Mutational Deterministic hypothesis (MD; Kondrashov 1988), which postulates that outcrossing will offer a net fitness advantage over selfing when the deleterious mutation rate per generation, U, is sufficiently large (~1.4) and only under conditions of synergistic epistasis (i.e. when the negative fitness impact of multiple deleterious mutations is greater than strictly additive) (Charlesworth 1990; Cutter and Payseur 2003). The strength of sexual selection has also been used to explain the maintenance of obligate outcrossing in the face of the reproductive advantage conferred by an asexual reproductive system (the so-called 'two-fold cost' of sex). Treatment of the subject has indicated that, all else being equal, if sexual selection causes deleterious mutations to have greater fitness impact on males, such mutations will be maintained in sexual populations at a lower equilibrium frequency than in asexual populations (Agrawal 2001; Siller 2001). Such a model may be extended into a comparison between dioecious and androdioecious species in that the reproductive advantage of the latter may be mitigated by the advantage of increased strength of sexual selection in the former. While most work in this field has been performed in the model research genus of *Drosophila*, such a system does not allow us the opportunity to directly compare the effects of sexual selection and the deleterious mutation rate between different mating types (i.e. dioecious *versus* androdioecious species).

The effect of mating type on genome evolution has been studied in some detail in plants where it is common to find closely related species with different mating types (e.g. *Arabidopsis thaliana* and *A. lyrata*). Such studies have found evidence that hermaphroditic species display reduced amounts of neutral polymorphism, higher levels of linkage disequilibrium and a generally reduced efficiency of selection relative to dioecious species, due in large part to increased Hill-Robertson interference in heavily inbred selfers (Charlesworth and Wright 2001; Glémin, Bazin, and Charlesworth 2006; Hill and Robertson 1966). However, estimates of U between *A. thaliana* and *A. lyrata* have produced values that are well below the threshold value of ~1.4 required to explain the maintenance of dioecy by the MD hypothesis alone (~0.22 – 0.58; Wright, Lauga, and Charlesworth 2002). It should be noted that these estimates have been calculated from a limited subset of genes as the whole genome sequence of *A. lyrata* has not yet been completed.

The nematode genus *Caenorhabditis* comprises both dioecious species (*C. remanei*, *C. brenneri*, *C. japonica*, etc.) as well as two known androdioecious species (*C. elegans* and *C. briggsae*). As the full genome sequences of *C. elegans*, *C. briggsae* and *C. remanei* are available, comparison of the patterns of evolution of genes among these

species provides an excellent opportunity to compare how different mating systems affect the broad patterns of genome evolution in animals. Numerous studies have shown that Sex and Reproduction Related (SRR) traits, particularly those involved in male reproductive function, evolve rapidly at both the morphological and genetic levels in a wide variety of taxa and that many of these genes evolve adaptively, indicating that their functions are beneficial to reproduction (Civetta and Singh 1998; Swanson and Vacquier 2002). Such observations are consistent with sexual selection in terms of female choice and competition for mates (especially sperm competition) acting as the primary driving force accelerating male evolution (Jagadeeshan and Singh 2005). Whole genome comparisons between these nematode species would allow us to examine the strength of sexual selection among different mating types by comparing the rates of evolution of SRR proteins. In addition these data also allow for more reliable estimates of U in *C. briggsae* and *C. remanei* than were previously possible through the use of comparative genomic data (Cutter and Payseur 2003).

To this end, we have generated a large dataset of three-way orthologous genes among theses species in order to compare the rates of evolution of a number of gene categories involved in SRR and non-SRR function. We find that genes expressed in sperm are evolving rapidly in both *C. remanei* and *C. briggsae*; however, sperm genes in *C. remanei* show a greater increase in their rate of evolution relative to other gene categories, providing evidence of stronger sexual selection in dioecy. We also use our dataset in order to obtain improved estimates of U in these species using previously established methods (Cutter and Payseur 2003; Eyre-Walker and Keightly 1999). In contrast, and unlike previous estimates of U, we find that hermaphrodites experience a significantly greater genomic rate of deleterious mutation as well as an overall greater rate of non-synonymous substitution. These results shed light on the role of sexual selection and deleterious mutation in the maintenance of sexual reproduction in nematodes.

2.3 Materials and Methods

Genomic Datasets

We obtained all unique, predicted peptides for *C. elegans* (release WS173), *C. briggsae* (release WS173) and *C. remanei* (release 11/29/2005) from the Wormbase FTP site (ftp://ftp.wormbase.org/pub/wormbase/genomes/). As the *C. remanei* predicted peptide dataset is known to contain redundant copies of genes due to heterozygosity in the sequenced genome, (E. Schwartz, pers. com.) we used Cluster Database at High Identity with Tolerance (CD-HIT; Li and Godzik 2006) in order to cluster and remove all additional transcripts that had greater than or equal to 98% sequence similarity at the protein level. The original dataset of 25,948 transcripts, was truncated down to 24,267 non-redundant transcripts that were used in further analysis.

INPARANOID (Remm, Storm, and Sonhammer 2002) was run with default parameters, using blastall version 2.2.14 with -VT emulation, on all three pairwise species comparisons. One-to-one best hit reciprocal orthologs were collected and clustered into three-way best-hit reciprocal orthologous trios. This method generated 11,594 orthologous gene trios of which 11,493 annotated transcripts were free of in-frame stop codons in all three species and thus could be used in further analysis.

Evolutionary Rate Estimates

The amino acid sequences of each orthologous trio were aligned with Dialign 2.2 using default parameters (Morgenstern 1999). The nucleotide sequences were then aligned with RevTrans 1.4 according to their corresponding protein alignments (Wernersson and Pedersen 2003). Non-synonymous (d_N) and synonymous (d_S) rates of divergence were computed for *C. briggsae* and *C. remanei* (using *C. elegans* as the outgroup species) using CODEML from PAML 3.15 (Yang and Nielsen 2002) under Model 0, in which a single rate was calculated for the entire phylogeny, as well as Model 1 in which a separate rate is estimated for each branch of the phylogeny.

Classification of Gene Functions

Genes were classified into functional categories based on the tissue/sex in which they display their highest level of expression according to Reinke et al. (2004). Genes were pooled into the following categories (with the number of genes in each category shown in brackets next to the name of the category itself): Sperm (361), Oocyte (622), Hermaphrodite (non-sperm, non- oocyte; 684), Male (non-sperm, non-oocyte; 289), Non-sex (non-sex biased, non-sperm, non-oocyte; 9537).

Calculation of Genomic Deleterious Mutation Rate

Rates of total mutation (M) and deleterious mutation (U) per generations were computed according to the procedure established by Eyre-Walker and Keightley (1999) and modified by Cutter and Payseur (2003): $M = Z(\Sigma L \times d_S)/ \Sigma L$, $U = M\text{-}Z(\Sigma L \times d_N)/ \Sigma L$, where L = gene length in nucleotide, $Z = 2$ (genomes) × number of genes × average gene length × (1/generation per year) × (1/divergence time). We used a genome size of 19,500 genes as per Stein et al.'s (2003) estimates, an average gene length of 1403.37 and 1414.62 for *C. briggsae* and *C. remanei* respectively computed from 10,822 genes with $d_S < 3$, 90 generations per year (Denver et al. 2000) and a divergence time of 44.5 million years (Cutter and Payseur 2003). U was also computed according to the neutral mutation rates proposed by Drake et al. (1998) (Supplementary Table 3). In order to take into account potential selection on synonymous sites, which would reduce the reliability of using d_S as a measure of the neutral mutation rate, we used a corrected measure of d_S using the Frequency of optimal codons (Fop, CodonW, http://codonw.sourceforge.net/)

taking into account the codon usage bias of *C. elegans* (Stenico, Lloyd, and Sharp 1994) for both *C. briggsae* (d_S = -1.6 × Fop + 1.527, $R^2 = 0.133$, $p < 2.2\times10^{-16}$) and *C. remanei* (d_S = -1.302 × Fop + 1.257, $R^2 = 0.094$, $p < 2.2\times10^{-16}$). We computed 95% confidence intervals from *U* using 1000 bootstraps (Manly 1991).

Statistics

All statistical analyses were performed using the R statistical package (R Development Core Team 2004). Permuted Kruskal-Wallis rank sum tests were performed with 10,000 permutations of the data using the 'coin' package.

2.4 Results

Faster Evolution of Sperm Proteins

We identified 11,493 gene trios free of in-frame stop codons that were orthologous between *C. elegans*, *C. briggsae* and *C. remanei* and were thus suitable for this analysis (Supplementary Table 1). Using Reinke et al.'s (2004) large-scale expression study in *C. elegans*, we classified each of the three-way orthologs into one of five functional categories (the number of genes in each category is shown in parentheses): Sperm (361), Oocyte (622), Hermaphrodite (684), Male (289), Non-sex (9537). Rates of synonymous substitutions per synonymous site (d_S) and non-synonymous substitutions per non-synonymous site (d_N) were estimated for all orthologs using PAML (Yang and Nielsen 2002) under model 0, allowing for a single rate among all branches (Supplementary Figure 1). Given the long estimates of divergence times between the three species, d_S was saturated (i.e. > 3 substitutions per site, Supplementary Table 1) and thus only d_N was used in further analysis of the entire dataset. We found that genes classified as having their highest level of expression in sperm according to Reinke et al. (2004) had a significantly higher d_N than other gene categories (Tukey Honestly Significant Difference [HSD] test, $p < 0.001$ in all comparisons, Supplementary Figure 1). In contrast, non-sex genes are evolving significantly slower than all other gene categories (Tukey HSD test, $p < 0.001$ in all comparisons). In order to control for a possible bias in our results due to saturation of d_S, we performed the analysis again, this time using only genes with $d_S < 3$. Our conclusions remain the same as sperm genes are showing a greater d_N than other gene categories (Tukey HSD test, $p < 0.001$ in all comparisons; Supplementary Table 2). Furthermore, using the truncated data set, we were able to compare the d_N/d_S ratio between gene categories, revealing a significantly higher d_N/d_S for both male and sperm genes as compared to other gene categories (Tukey HSD test, $p < 0.001$ in all comparison). These two classes were not significantly different from one another ($p = 0.986$).

We repeated the analysis of rates of evolutionary divergence, this time calculating a separate d_N for each branch of the phylogeny (model 1 in PAML). There was a significantly higher median d_N in the branch leading to *C. briggsae* than in the branch leading to *C. remanei* (median d_N of 0.0459 and 0.0346 for *C. briggsae* and *C. remanei* respectively; Kruskal-Wallis rank sum test, 10,000 replicates $p < 2.2\times10^{-16}$). Sperm genes evolve significantly more rapidly than all other gene categories in *C. remanei* (Tukey HSD test, $p < 0.001$ in all comparisons) while in *C. briggsae* sperm genes are showing a significantly higher d_N than non-sex genes and male genes ($p < 0.001$ in both comparisons) but do not differ from hermaphrodite genes ($p = 0.293$), nor oocyte genes ($p = 0.4$). However, there was no significant difference in the evolutionary rates of sperm genes ($p = 0.998$) nor male genes ($p = 0.247$) between mating types (i.e. dioecy *versus* androdioecy; Figure 1). When limiting the analysis to genes showing $d_S<3$ in both branches, our conclusions remain the same (Supplementary Table 2).

Accelerated Sperm Evolution in Dioecy

Previous mutational models predict that hermaphroditic species should have a higher rate of fixation of slightly deleterious mutations due to inbreeding, leading to reduced effective population size (Charlesworth 1990), the results of which could lead to a higher rate of substitutions in hermaphrodites and thus the higher overall d_N observed in *C. briggsae*. With this in mind, we sought to determine whether the degree of accelerated evolution of sperm proteins relative to other gene classes was similar for both mating types using an average centering approach and, in doing so, control for the effect of a higher rate of non-synonymous substitution in hermaphrodites. We compared only the residuals of the d_N estimates for each gene category (i.e. the value of the d_N for each gene from which is subtracted the mean d_N among all genes within that species), allowing us to determine the degree to which the d_N for each category diverges from the species average. Pooling the residuals of the d_N into sperm and non sperm categories, and performing an analysis of variance on these two groups while taking into account the effect of mating type, we observed a significant interaction between mating type and the variation of d_N between the two gene categories ($F_{1,22982} = 4.7937$, $p = 0.02857$), indicating a faster rate of evolution for sperm genes relative to non-sperm genes in the dioecious species, *C. remanei,* when compared to the androdioecious *C. briggsae* (Figure 2.1 inset). In order to rule out any potential bias in our data due to the long divergence times between species, we also repeated the ANOVA using only genes with $d_S < 3$. Our conclusions remain the same ($F_{1,21640} = 5.1462$, $p = 0.02334$).

Higher Genomic Rate of Deleterious Mutations in Hermaphrodites

In order for the MD hypothesis alone to explain the maintenance of dioecy against invasion from reproductively advantageous hermaphrodites, the deleterious mutation rate per genome per generation, U, would have to be on the order ~1.4 (Cutter and Payseur

2003). Furthermore, as *U* itself appears to be an adaptive trait (Bjedov et al. 2003; Baer et al. 2005), we would predict that the deleterious mutation rate per genome per generation would be higher in dioecious species as compared to related androdioecious species. A previous study attempted to compare the genomic deleterious mutation rate, *U*, between *Caenorhabditis* species using a small sample of genes and found no significant differences between mating types (Cutter and Payseur 2003). Using our whole-genome ortholog dataset, we attempted to obtain a more accurate estimate of *U* for both *C. briggsae* and *C. remanei* using a modified version of Cutter and Payseur's (2003) method (see materials and methods). We used the following parameters when estimating *U*: 19,500 genes in both species (Stein et al. 2003), an average gene length of 1403.37 and 1414.62 nucleotides for *C. briggsae* and *C. remanei* respectively, a divergence time of 44.5 million years between the two species (Cutter and Payseur 2003), and 90 generations/year. Under these conditions, we find that *C. briggsae* has a significantly higher *U* than *C. remanei* (0.01104 with 95% CI: 0.0108 – 0.0112 and 0.00962 with 95% CI: 0.0094 – 0.0097; alternate values of *U* calculated from a variety of biologically realistic parameters are presented in Supplementary Table 3).

Effect of Chromosome Position on Gene Evolution

Previous studies have shown that chromosomal position can have a significant effect on the rates of evolution of genes, especially those involved in SRR function (Torgerson and Singh 2003, 2006; Stevison, Counterman and Noor 2004). Several studies have found that sex-chromosomes and autosomes can display markedly different rates of evolution due to the unique, sex-dependent patterns of transmission of the former (Charlesworth, Coyne, and Barton 1987; Torgerson and Singh 2003; Counterman, Ortíz-Barrientos, and Noor 2006; Musters, Huntley, and Singh 2006; Mank, Axelsson, and Ellegren 2007). However, both *C. elegans* and *C. briggsae* reproduce overwhelmingly by self-fertilization and we should expect the sex chromosome, X, to behave like an autosome as it spends the majority of its time in the homogametic state. Because the *C. remanei* genome has not yet had contigs assigned to chromosomes, we determined the chromosomal positions for all of the three-way orthologous genes for which data was available in *C. briggsae* and *C. elegans* (Bieri et al. 2007). Gene synteny information was available for a total 10,304 genes from our data set, of which 403 do not present conserved synteny between the two species and 1189 genes are not assigned to chromosomes in *C. briggsae*. We first compared the rate of evolution of genes between different chromosomes for the genes whose synteny was conserved between the two species. As expected, we were unable to detect a significant difference between the X chromosome as compared to the autosomes in the average rate of non-synonymous substitution in genes (Tukey HSD test, $p > 0.05$ in each comparison), with the exception of chromosome IV, which is showing a significantly lower d_N than genes on chromosomes X, I and V ($p = 0.0354$, 1.6×10^{-4} and 7.63×10^{-3} respectively). Among the genes whose synteny was not conserved between *C. elegans* and *C. briggsae*, we

determined whether any of the functional categories were over-represented. We found that sperm genes are significantly over-represented among genes with non-conserved synteny ($chi^2 = 425$, $df = 1$, $p = 0$, Table 2) while non-sex genes are significantly under-represented among these ($chi^2 = 23.37$, $df = 1$, $p = 1.3\times10^{-6}$, Table 2.2). The differences between other categories were non-significant. Genes lacking conserved synteny are also presenting a significantly higher d_N (under Model 0) than genes with conserved synteny (Kruskal-Wallis rank sum test, $p < 2.2\times10^{-16}$). Removal of sperm genes from this analysis does not change the results ($p < 2.2\times10^{-16}$), indicating that this phenomenon is not simply due to an enrichment of rapidly evolving sperm genes.

2.5 Discussion

Evolution of Sperm Proteins

Accelerated evolution of spermatogenesis proteins is believed to result from sexual selection in the form of sperm competition and gametic interactions (Jagadeeshan and Singh 2005; Singh and Kulathinal 2005). Theoretical modeling has suggested that the opportunity for sexual selection, and thus the strength of selection itself, is reduced in hermaphroditic species as compared to dioecious species, due primarily to reduced competition for mates among self-fertilizing hermaphrodites (Greeff and Michiels 1999). Despite these considerations, we find evidence for a persistent signal of accelerated evolution among genes involved in spermatogenesis within the lineage leading to *C. briggsae* (Figure 2.1). A previous study also found that sperm genes evolve more rapidly than other gene categories when performing a pairwise analysis between *C. briggsae* and *C. elegans* (Cutter and Ward 2005). Such observations could result from two possible evolutionary scenarios, neither of which is mutually exclusive:

It is possible that in natural populations of *C. briggsae*, sufficient sperm competition occurs to drive the rapid evolution of spermatogenic proteins. It is unlikely that gametic competition within individual selfers could occur as hermaphrodites are presenting near 100% efficiency of sperm usage (Ward and Carrel 1979). However, sexual selection will favor efficient male sperm in order to outcompete those of the hermaphrodite upon successful mating (Chasnov and Chow 2002). While the frequency of outcrossing should be low in natural populations of *C. briggsae* (Cutter et al. 2006) there may still remain enough opportunity to produce a detectable signal of selection among sperm proteins. Alternatively, the rapid evolution of spermatogenic proteins within hermaphrodites could also be explained if androdioecy has evolved recently within the lineage leading to *C. briggsae*. In this case our results would reflect the accelerated evolution of sex genes within the dioecious ancestor to *C. briggsae* (Cutter and Payseur 2003), the signal of which remains detectable in the present hermaphroditic state. Such a possibility is further supported by evolutionary studies performed in plants which suggest that despite the reproductive advantages acquired by evolving hermaphrodism, this

mating type may ultimately prove to be an evolutionary 'dead-end' (Takebayashi and Morrell 2001). If hermaphrodite genomes have reduced efficiency of natural selection and thus reduced efficiency in adaptation, as theory predicts, this mating type could be difficult to sustain over long periods of evolutionary time. Thus, while hermaphrodism may evolve frequently in some lineages, it may not be evolutionarily stable over long periods. The extremely low frequency of males in natural populations of *C. briggsae*, coupled to the reduced opportunity for sexual selection in androdioecious species, would suggest that accelerated evolution of sperm proteins is most likely a remnant of a recent ancestral, male-female state of the *C. briggsae* lineage.

As shown in Table 2.1, *C. briggsae* has a higher rate of non-synonymous substitution than does *C. remanei* in most gene categories, with the exception of male genes and those involved in spermatogenesis. The increased rate of non-synonymous substitution observed in *C. briggsae* may be the result of two non-exclusive processes: Firstly, the rate of fixation of slightly deleterious amino acid substitutions is predicted to be higher in non-outcrossing species relative to obligate outcrossers due to interference of selection between closely linked loci, the 'Hill-Robertson effect' (Hill and Robertson 1966; Ohta 1973). As most amino acid substitutions are presumed to be slightly deleterious (Kimura 1968), a higher d_N is to be expected in hermaphrodites as compared to dioecious species. Secondly, it is possible that *C. briggsae* has evolved an increased genomic mutation rate relative to that of *C. remanei*. Experimental evidence suggests that the mutation rate of *C. briggsae* may be higher than that of *C. elegans* (Ostrow et al. 2007), however there is no experimental evidence indicating that *C. briggsae* experiences a higher genomic mutation rate than *C. remanei* (Cutter and Payseur 2003). Therefore a comparison of the rates of evolution of gene categories between species of different mating types would require an appropriate correction for the expected differences in the rate of non-synonymous substitution. We find that, *C. remanei,* shows a greater acceleration in the rate of evolution of proteins expressed in sperm relative to other tissues (Figure 2.1) than does *C. briggsae*. Such an observation is consistent with previous, theoretical predictions that species with separate males and females should be exposed to stronger sexual selection pressure than simultaneous hermaphrodites (Greeff and Michiels 1999).

Effect of Chromosome Position and Synteny on Gene Evolution

We observed a significant over-representation of genes involved in spermatogenesis among those that are non-syntenic between *C. elegans* and *C. briggsae* (Table 2.2). These results support previous studies in other phyla that found a significant enrichment of testis-expressed genes among those that are translocated as well as a high frequency of retrotransposition of genes off of the X chromosome (Betrán, Thornton, and Long 2002; Emerson et al. 2004). Despite their current hermaphroditic status, it would appear that these species were subject to similar selective pressures as other dioecious

taxa, which act to translocate sex-biased genes into more favorable positions within the genome (Miller et al. 2004; Kulathinal and Singh 2005). It is unlikely that such pressure would continue to act in the context of hermaphrodism, which implies that these observations reflect pressures that existed in an ancestral dioecious state. Coupled to the observation of a persistent signal of rapid evolution of sperm proteins, this suggests a relatively recent origin of hermaphrodism in these species. The discovery of a new dioecious species, *JU727*, more closely related to *C. briggsae* than *C. remanei* (Kiontke and Sudhaus 2006) further supports this hypothesis.

Comparison of Deleterious Mutation Rate Between Mating Types

Given the reproductive advantages possessed by hermaphrodites over dioecious populations (Charlesworth 1990; Cutter 2005), the persistence of separate males and females calls for an explanation. Several hypotheses have been forwarded in order to explain the evolution and maintenance of different mating types. The Mutationally Deterministic (MD) hypothesis argues that, within the context of synergistic epistasis, dioecy is maintained due to its efficiency at purging deleterious mutations (Kondrashov 1988). In order for the MD hypothesis to explain the maintenance of separate sexes, it would require that dioecious species experience a greater U than hermaphrodites, and be above a threshold of ~1.4 (Kondrashov 1988, Cutter and Payseur 2003; Lloyd 1980). Cutter and Payseur's (2003) small-scale analysis of ten orthologous loci between *C. elegans*, *C. briggsae* and *C. remanei* found no significant differences in U, nor the rates of evolution of genes between the three species. Using our whole-genomic dataset, we found a significant difference in U between the two mating types; however, it was in the opposite direction from theoretical expectations. Our estimates of U in both species (~0.01 - ~0.5; see Supplementary Table 3) are between one and two orders of magnitude lower than those required for the MD hypothesis alone to explain the maintenance of obligate outcrossing in *C. remanei*. Our observation of a greater U in hermaphrodites also contradicts previous theoretical expectations suggesting that the change to hermaphroditism from dioecy would lead to the evolution of reduced deleterious mutation rates in order to slow the accumulation of deleterious substitutions (Birky 1999; West, Lively, and Read 1999). However, under the assumption that hermaphrodism is of recent origin in *C. briggsae*, such a reduction in mutation rate could potentially occur in the future.

It should also be noted the *C. remanei* genome appears to be ~15-30% larger than that of *C. briggsae* (Stein et al. 2003) (http://genome.wustl.edu/pub/organism/Invertebrates/Caenorhabditis_remanei/assembly/Caenorhabditis_remanei-15.0.1/ASSEMBLY), which could imply that it also may have more genes than the latter. While this remains to be determined, a larger amount of genes in one species would affect estimates of U and could lead to opposite results (i.e. *C. remanei* having a statistically significantly higher U than *C. briggsae*; Supplementary

Table 3). In terms of magnitude, our estimates of U agree with those of Cutter and Payseur's (2003) study as well as that of Baer et al. (2006); however, they are up to ~50 times lower than those computed by Denver et al. (2000) in *C. elegans* from direct sequencing of mutation accumulation lines. A variety of possibilities could explain such a discrepancy, including variation due to parameter estimates (i.e. divergence time and number of generations per year) as well as error associated with using d_S as a measure of the neutral mutation rate (Halligan et al. 2004). It should also be noted that, as previously indicated, estimates of d_S between *C. remanei* and *C. briggsae* were saturated and therefore could also lead to inaccurate estimates of U. However, reducing the data set such that we calculated U only for genes with d_S lower than 3 (or lower than 1) produced very similar results, suggesting that the saturation of d_S at some loci is not significantly biasing our estimates of U (Supplementary Table 3). Regardless, after taking into account these considerations U remains under the threshold of 1.4 under a variety of biologically realistic parameters.

Sexual reproduction varies widely in form and expression from single celled eukaryotes to sexually dimorphic organisms with exaggerated differences in sexual traits between closely related species. In the past, the leading population genetics theories as to the benefits of sex have emphasized recombination and mutation purging, and thus could apply to all sexual organisms – regardless of mating type or system (Bell 1982; Kondrashov 1988). Varying degrees of sexual dimorphism apply to almost all multi-cellular animals. Its intricate association with the process of sexual reproduction itself would suggest a causal association with the maintenance of sex; one that has recently been made explicit in theoretical form (Agrawal 2001; Siller 2001). Sexual selection has the potential to drive rapid evolution, not only via increased opportunity for mutation purging due to increased variance in male mating success, but also by direct effect of positive selection resulting selection on traits which are beneficial to the reproductive success of individual sexes. The aggregated effect of all of these beneficial properties derived from the opportunity for sexual selection under the common situation of sexual dimorphism may contribute to paying the 'two-fold' cost of sex, which theories focusing on recombination and mutational purging seem to be unable to do alone (Crow and Kimura 1970; Cutter and Payseur 2003).

In conclusion, we find evidence for stronger sexual selection pressure in the dioecious *C. remanei* as compared to the androdioecious *C. briggsae* as evidenced by its increased rate of spermatogenesis-related gene evolution relative to other gene categories. We have also used our whole-genome dataset in order to compare estimates of the deleterious mutation rate per generation among these two mating types and find that our estimates are on the order of two orders of magnitude below what would be required for the MD hypothesis to explain the maintenance of dioecy alone. Despite these considerations, environmental constraints, parasitism and sexual selection have also been suggested to explain the maintenance of dioecy against invasion of hermaphrodites and/or asexuals (Bell 1982; Agrawal 2001; Siller 2001). Our study provides evidence that

efficient sexual selection and dioecy are tightly linked within nematodes. Comparisons of different mating systems using a greater number of organisms as well as representative taxa will be required to determine whether this is a general phenomenon among sexually reproducing metazoans. If sexual selection is indeed important in the maintenance of obligate outcrossing in *Caenorhabditis*, we are left with an intriguing paradox: does loss of efficient sexual selection precede the evolution of hermaphrodism or does evolution of hermaphrodism precede relaxation of sexual selection? Further experimental data and theoretical modeling will be required to answer such questions.

Acknowledgements

We are grateful to Aneil Agrawal, Asher Cutter, Ben Evans, and two anonymous referees for the comments they raised on the early version of this manuscript. This work was funded by a National Sciences and Engineering Research Council of Canada (NSERC) Post-Graduate Doctoral Scholarship to CGA and NSERC grants to RSS and BPG.

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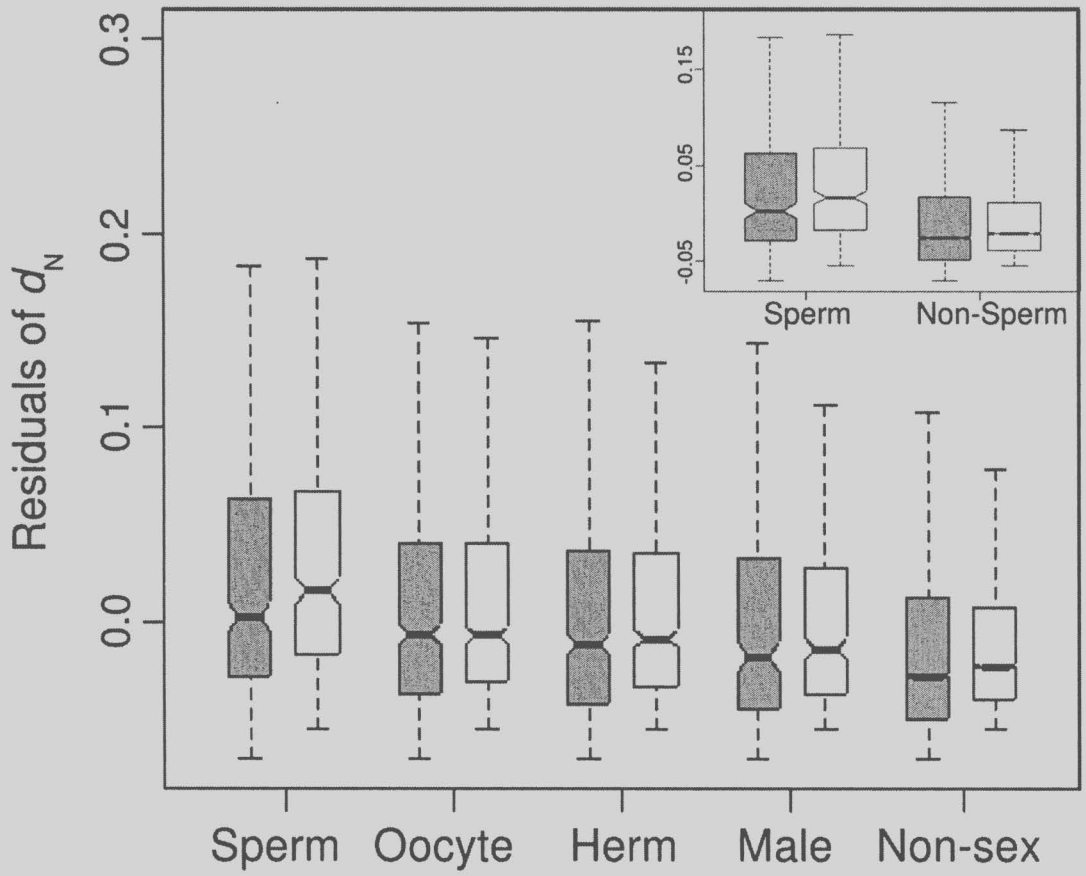

Figure 2.1: Box-plot comparing the residuals of d_N along the *C. remanei* and *C. briggsae* branches under model M1 between gene categories. Inset shows the comparison of sperm genes to all other genes (non-sperm). *C. briggsae* categories are represented in gray, while *C. remanei* categories are represented in white. Category abbreviations are as follows: Hermaphrodite (Herm), Non-sex (non-sex biased, non-sperm, non-oocyte).

Table 2.1: Average d_N (95% confidence interval limits) for genes classified according to Reinke et al (2004). d_N was calculated for the whole tree (model 0) and for each branch of the phylogeny (model 1) using PAML (17). Category abbreviations are as follows: Non-sex (non-sex biased, non-sperm, non-oocyte).

	N	d_N model 0	d_N *C. briggsae*	d_N *C. remanei*
Hermaphrodite	684	0.255 (0.238-0.273)	0.085 (0.079-0.091)	0.071 (0.065-0.077)
Male	289	0.241 (0.215-0.267)	0.077 (0.068-0.085)	0.062 (0.054-0.070)
Non-sex	9537	0.198 (0.194-0.202)	0.067 (0.066-0.069)	0.051 (0.049-0.052)
Oocyte	622	0.264 (0.246-0.281)	0.086 (0.080-0.091)	0.072 (0.067-0.077)
Sperm	361	0.343 (0.319-0.368)	0.095 (0.087-0.102)	0.090 (0.083-0.98)

Table 2.2: Number of genes (percentage of total genes in the category) presenting non-conserved synteny between *C. elegans* and *C. briggsae* among the different gene categories. The proportion of genes in each category with non-conserved synteny was compared to the expected number using a chi^2 test (df = 1).

	Hermaphrodite	Male	Non-sex	Oocyte	Sperm
Non-conserved synteny	32 (4.68)	14 (4.84)	246 (2.58)	25 (4.02)	86 (23.82)
chi^2 (p-value)	2.68 (0.102)	1.48 (0.224)	23.37 (1.3×10^{-6})	0.47 (0.493)	425 (0)

CHAPTER 3

Association Between Levels of Coding Sequence Divergence and Gene Misregulation in *Drosophila* Male Hybrids

This chapter has been published in the Journal of Molecular Evolution:

Artieri Carlo G.*, Wilfried Haerty*, and Rama S. Singh. 2007. Association Between Levels of Coding Sequence Divergence and Gene Misregulation in Drosophila Male Hybrids. *Journal of Molecular Evolution* 65, no. 6 (December): 697-704.

In this article, we used data generated from a large-scale *Drosophila* interspecific hybrid expression profiling experiment in order to test a) if divergence in gene expression levels is correlated with sequence divergence between species and b) if the degree to which genes are improperly expressed in hybrids is correlated with coding sequence divergence between the parental species. We hypothesized that selective pressures known to act at the level of coding sequence divergence (e.g., sexual selection) could also act to drive divergence of expression levels as well. W. Haerty and I conceived of the study and collaborated in analyzing the data. We also shared the duty of drafting the manuscript and all three authors assisted in final editing and revisions. In order to indicate the collaborative nature of this work, the asterisks (*) in the reference above indicate that both W. Haerty and I contributed equally to this manuscript. Please note that supplementary materials can be found online at http://www.springer.com/life+sci/cell+biology/journal/239/.

3.1 Abstract

Previous studies have shown widespread conservation of gene expression levels between species of the *Drosophila melanogaster* subgroup as well as a positive correlation between coding sequence divergence and expression level divergence between species. Meanwhile, large-scale misregulation of gene expression level has been described in interspecific sterile hybrids between *D. melanogaster*, *D. simulans*, *D. mauritiana* and *D. sechellia*. Using data from gene expression analysis involving *D simulans*, *D. melanogaster* and their hybrids, we observed a significant positive correlation between protein sequence divergence and gene expression differences between hybrids and their parental species. Furthermore, we demonstrate that under-expressed misregulated genes in hybrids are evolving more rapidly at the protein sequence level than non-misregulated genes or over-expressed misregulated genes, highlighting the possible effects of sexual and natural selection as male-biased genes and non-essential genes are the principle gene categories affected by interspecific hybrid misregulation.

3.2 Introduction

Recent comparative gene expression analyses between closely-related species of the *Drosophila melanogaster* subgroup have highlighted the importance of stabilizing selection in shaping the evolution of gene expression within (Rifkin et al. 2005) and across species (Rifkin, et al. 2003; Lemos et al. 2005; Gilad et al. 2006). For instance, using a mutation-drift model to infer patterns of evolution of gene expression levels across *D. melanogaster*, *D. simulans* and *D. yakuba*, Rifkin, et al. (2003) found that 67% of genes demonstrated similar levels of expression among all three species. Despite the observation of widespread conservation of expression levels between species, the sterile offspring of interspecific crosses within the *D. melanogaster* subgroup demonstrate large-scale misregulation of gene expression relative to parental expression levels (Ranz et al. 2004; Michalak and Noor 2003; 2004; Haerty and Singh 2006; Moehring et al. 2007). This suggests that, while gene expression remains conserved between species, nucleotide sequence divergence of regulatory elements is occurring; only in the case of interspecific hybridizations are the effects of such nucleotide divergence revealed.

The phenomenon of interspecific hybrid sterility is thought to arise from genetic incompatibilities linked to divergence at interacting loci (Dobzhansky 1936, Muller 1942). Classical genetic studies of hybrid sterility in multiple taxa have supported the Dobzhansky-Muller model at the gene-gene interaction level (for review see Coyne and Orr 2004). The phenomenon of gene misregulation in interspecific sterile hybrids has provided evidence of Dobzhanky-Muller incompatibilities at the transcriptional level as well (Ortiz-Barrientos et al. 2006). Michalak and Noor (2004) have postulated a causal link between gene misregulation and hybrid sterility. In a study of the expression patterns

of a small number of genes known to be misregulated in the interspecific hybrids between *D. simulans* and *D. mauritiana*, the authors found that four of the five genes assayed were misregulated in sterile fifth-generation backcross males while fertile fifth-generation backcross males demonstrated parental levels of expression for these same genes. Classical and molecular genetic analyses have also found that genes implicated in hybrid sterility or inviability show evidence of rapid and adaptive evolution at the nucleotide level (Ting et al. 1998; Presgraves et al. 2003; Barbash et al. 2004; Presgraves and Stephan 2007). As protein coding sequence evolution and gene expression divergence appear to be coupled (Castillo-Davis et al. 2004; Nuzhdin et al. 2004; Lemos et al. 2005), we sought to test if the genes that are misregulated in hybrids show a greater degree of protein sequence divergence relative to non-misregulated genes. We find that genes that are misregulated in *D. simulans* female × *D. melanogaster* male hybrids (specifically those under-expressed relative to parents) are evolving more rapidly at the amino acid level than non-misregulated genes. In addition, misregulated genes show a paucity of proteins with known lethal mutant phenotypes, suggesting that similar selective forces are acting to minimize sequence and expression divergence for essential genes.

3.3 Materials and Methods

Using cDNA microarray hybridization data from a study of gene expression in hybrid testes between *D. simulans* females and *D. melanogaster*, *D. mauritiana* and *D. sechellia* males (Gene Expression Omnibus databank accession number GSE3673; Haerty and Singh 2006), genes were classified as misregulated or non-misregulated in hybrids in comparison to both parents as well as whether parental expression levels were significantly different from one another as per the criteria used in Haerty and Singh (2006). The absolute average difference in the Log_2 ratio (expression in the testis / expression in *D. melanogaster* whole body) of the expression values was computed as a measure of gene expression difference between *D. melanogaster* – *D. simulans* as well as between parents and hybrids.

Using the *Drosophila* genome annotation project (http://rana.lbl.gov/drosophila/, Supplementary Table 1), we were able to retrieve coding sequences for *D. melanogaster* and *D. simulans* for a total of 2637 of the genes expressed within the hybrids between *D. simulans* females and *D. melanogaster* males in Haerty and Singh's (2006) study (Supplementary Table 1). The longest available transcript for each gene was used. Sequences were aligned using ClustalW (Thompson, Higgins and Gibson 1994) according to the protein sequence alignment. Non-synonymous (d_N) and synonymous (d_S) rates of divergence were computed using CODEML from PAML (Yang and Nielsen 2002). Using available gene predictions for *D. melanogaster*, *D. simulans*, *D. sechellia*, *D. yakuba*, *D. erecta* and *D. ananassae*, we tested for evidence of positive selection for each gene using models allowing d_N/d_S to vary across sites (M7 and M8, PAML; Yang

and Nielsen 2002; Larracuente et al. submitted). A Bonferroni correction was applied to the results. Using FlyBase annotations (www.flybase.org) we collected information on lethal and sterile mutant phenotypes for all of the genes used in our study.

Previous studies have shown that potential hybridization bias can arise from the use of a single species microarray for cross species hybridization due to sequence divergence between species (Gilad et al. 2005; Oshlack et al. 2007). Therefore we applied a more conservative procedure by limiting our analysis to genes without significant expression difference between *D. melanogaster* and *D. simulans* in order to account for the possible confounding effect of sequence divergence on gene hybridization (Michalak and Noor 2003). After removing genes with significant differences in expression levels between *D. melanogaster* and *D. simulans,* 1841 out of 2637 genes remained, including 729 genes significantly misregulated in comparison to both parental species (245 over-expressed, 484 under-expressed).

The differences between significantly and non-significantly misregulated genes were determined using permuted Kruskal-Wallis rank sum tests (10000 permutations, coin package for R; R Development Core Team 2004). Kendall rank sum coefficients of correlation between sequence divergence and expression difference were computed for coding sequences using d_N, d_S and d_N/d_S values. As a significant correlation exists between d_N and the expression differences between parental species as well as between d_N and the expression differences between parental species and the hybrids, we controlled for any potential effect of the former on the latter using multiple regression analysis. Our regression model was: lm (d_N~*mel* (or *sim*) + parents) where *mel* represents the expression difference between *D. melanogaster* and the hybrids, *sim* the difference between *D. simulans* and the hybrids and parents the expression difference between parental species. Over representations of genes with lethal or sterile mutant phenotypes relative to expectations within categories were computed using chi-square tests.

3.4 Results

Faster sequence divergence of under-expressed misregulated genes in hybrids

Using expression data for the hybrids between *D. simulans* females and *D. melanogaster* males obtained from Haerty and Singh (2006), we compared the rate of evolution between misregulated and non-misregulated genes in hybrids and analyzed the relationship between nucleotide sequence divergence and gene expression differences between hybrids and parental species. We found that genes misregulated in hybrids show greater d_N, and d_N/d_S relative to non-misregulated genes (Kruskal-Wallis test, $p < 2\times10^{-16}$ in both comparisons; Supplementary Table 2) while no significant differences were observed for d_S (Kruskal-Wallis test, $p = 0.063$). More specifically, when comparing over- and under-expressed misregulated genes in hybrids, we observed that under-expressed genes show greater d_N, and d_N/d_S than over-expressed genes (Kruskal-Wallis

test $p < 2.2\times10^{-16}$ in both comparisons, a Bonferroni correction was applied; Figure 3.1; Supplementary Table 2), in fact, over-expressed genes are showing significantly lower d_N, and d_N/d_S than non-misregulated genes (Kruskal-Wallis test, p = 0.0012 and p = 0.0024 respectively, a Bonferroni correction was applied). Again, no significant differences were observed for the d_S between non-misregulated genes and over-expressed or under-expressed misregulated genes nor between over and under-misregulated genes (Kruskal-Wallis test, p = 0.6891, p = 0.2494, p = 0.5876 respectively). Given that over- and under-expressed misregulated genes show differences in their evolutionary patterns, we divided them into two separate categories for all subsequent analyses.

Supporting the results of previous studies (Castillo-Davis, Hartl and Achaz 2004; Lemos et al. 2005), we also found that genes with divergent expression between species had significantly greater d_N (Kruskal-Wallis test p= 0.013) and d_N/d_S (Kruskal-Wallis test p= 0.039) than genes with similar expression levels between species (Figure 3.1; Supplementary Table 2). Differences in d_S were non-significant (Kruskal-Wallis test, p= 0.964). We attempted to determine whether a greater proportion of misregulated genes show evidence of positive selection than do non-misregulated genes. Unfortunately, only 17 genes in our entire dataset display evidence of positive selection after the application of Bonferroni correction, preventing the application of reliable statistical analysis.

We attempted to control for any potential hybridization bias of *D. simulans* transcripts on the *D. melanogaster* array by removing genes showing significantly different levels of expression between parental species (as per Michalak and Noor 2003). We observed similar results to the analysis using the full dataset, as under-expressed misregulated genes present a greater d_N and d_N/d_S than non-misregulated or over-expressed misregulated genes (Kruskal-Wallis test, $p < 2.2\times10^{-16}$ in all comparisons, a Bonferroni correction was applied, Table 3.1). Also, as previously observed, over-expressed misregulated genes present a lower d_N and d_N/d_S than non-misregulated genes (Kruskal-Wallis test, p = 0.0075 and p = 0.0089 respectively, a Bonferroni correction was applied). No significant difference was observed for the d_S between non-misregulated and over-expressed or under-expressed misregulated genes (Kruskal-Wallis test, p = 0.4849 and p = 0.2559) nor between over-and under-misregulated genes (p = 0.5893).

Correlation between sequence divergence and misregulation in hybrids

In order to test for a possible association between gene misregulation and sequence divergence at the protein level, we performed a correlation analysis between the estimated rates of nucleotide sequence evolution and the absolute, average gene expression differences between parents and between hybrids, and both parental species. We found a significant correlation between d_N and d_N/d_S and gene expression differences between species as well as between parents and their hybrids (Figure 3.2). d_S was only significantly correlated with gene expression differences between the *D. melanogaster* parent and the hybrids, which may reflect the greater expression level detection ability of

these transcripts on the *D. melanogaster* cDNA microarray. Removing the effect of parental expression differences, we still observed a significant relationship between expressions difference between *D. melanogaster* and the hybrids and d_N or d_N/d_S ($p < 2.2 \times 10^{-16}$ and $p < 2.2 \times 10^{-16}$ respectively) and between expression differences between *D. simulans* and the hybrids for d_N or d_N/d_S ($p = 9.17 \times 10^{-12}$ and $p = 2.18 \times 10^{-8}$ respectively). Furthermore, the absence of significant correlation between d_S and expression difference between *D. melanogaster* and *D. simulans* (Figure 3.2) indicates that the previously observed significant correlations between d_N and expression differences between parental species and their hybrids are not the results of hybridization biases caused by sequence divergence.

Once again, in order to remove any potential bias linked to sequence divergence, we reanalyzed the data, removing genes showing a significant expression differences between parental species. The conclusions of the analysis remain the same (Table 3.2).

Functional difference between non-misregulated and misregulated genes in hybrids

Nuzhdin et al. (2004) found that genes with known mutations of large phenotypic effect were under-represented in the category of genes with divergent expression between species as compared to genes with similar levels of expression. We performed an analysis in which we determined whether genes with known lethal or sterile mutant phenotypes showed the same distribution between misregulated and non-misregulated genes in hybrids. We found an under representation of genes with known lethal mutant phenotypes among the misregulated genes in hybrids (84/978 vs. 205/1659, $chi^2 = 7.25$, $p = 0.007$). When examining the distribution of lethal phenotypes and taking into account the pattern of misregulation in hybrids, a significant under-representation of genes with lethal mutant phenotypes in under-expressed misregulated genes as compared to non-misregulated or over-expressed misregulated genes is observed (50/696 vs. 205/1659, $chi^2 = 11.15$, $df = 1$, $p = 8.4 \times 10^{-4}$ and 50/696 vs. 34/280, $chi^2 = 5.15$, $df = 1$, $p = 0.0232$ respectively). We found no significant difference between non-misregulated and over-expressed misregulated genes (205/1659 vs. 34/280 $chi^2 = 0.01$, $df = 1$, $p = 0.92$). There was also no significant difference in the proportion of genes with known sterile mutant phenotypes between misregulated and non-misregulated genes in hybrids ($chi^2 = 1.8$, $p = 0.180$). However, only 29 genes in our dataset had annotated male specific sterile mutant phenotypes, reducing the power of our statistical analysis.

3.5 Discussion

Consideration of hybridization bias of interspecific transcripts on D. melanogaster microarray

No correlation was observed between the rate of synonymous substitution (d_S) and magnitude of expression difference between species in our data set, indicating that the effect of sequence divergence on hybridization efficiency may be small. Moreover, removing all genes showing a significant difference in expression level between parental species (as per Michalak and Noor 2003) does not affect the conclusions of the previous analysis. As noted by the study of Gilad et al. (2005), performing cross species hybridizations on a single species array for the purpose of direct comparison of relative expression levels can lead to biased estimates, due to the effect of sequence divergence on the efficiency of hybridization. However, in the same study it was also found that when a minimum between species expression difference cut-off of 1.5 fold was employed, almost all of the genes classified as differentially expressed on a single species array were confirmed by multi-species array analyses. In the present data set, the smallest gene expression difference between *D. melanogaster* and *D. simulans* for genes that were considered significantly differentially expressed is 1.59 fold. In the case of the comparisons between the hybrids and the parental species the smallest gene expression difference for genes classified as significantly misregulated is 1.02 fold (a total of 7 genes out of 978 show a gene expression difference smaller than 1.5).

Similar interpretations of cross-species hybridizations have also been corroborated by the recent study of Moehring et al. (2007) on gene expression in interspecific hybrids between *D. simulans*, *D. sechellia* and *D. mauritiana.* The authors of this study compared the accuracy of their results from cross species hybridization on a single species microarray to a small-scale multi-species array and showed that, although cross species hybridizations lead to a decrease of power to detect genes significantly differentially expressed between species, genes called significantly misregulated on the *D. melanogaster* single species array were also observed to be significantly misregulated on the multi-species microarray.

Under-expressed misregulated genes diverge more rapidly

As the comparison between parental species and hybrids was performed on genes expressed in the testes, a possible tissue effect (i.e. faster evolution of testis expressed genes in comparison to genes expressed in different organs, Civetta and Singh 1995; Jagadeeshan and Singh 2005) should not account for the increased divergence observed among misregulated genes when compared to non-misregulated genes in hybrids. The greater average d_N and d_N/d_S coupled with the absence of significant differences for the average d_S in misregulated genes suggests that the observed sequence divergence may be due to directional selection. Unfortunately, as we implemented a conservative test for

positive selection (comparison of models M7 and M8 from PAML, associated with Bonferroni correction) very few genes within our dataset show significant evidence of positive selection (i.e. $d_N/d_S > 1$; 17 genes in total; Supplementary Table 3). Therefore, we were unable to test whether misregulated genes showed a significant enrichment of genes demonstrating evidence of Darwinian selection. However, it should be noted that the rapid divergence of misregulated genes is also consistent with the predictions of models of the accumulation of Dobzhansky-Muller incompatibilities under directional selection (Johnson and Porter 2000; 2007). Such simulations have found that the rate of accumulation of incompatibilities between populations, and thus the rate of speciation, increases when the affected loci are under directional selection.

An alternative explanation for rapid evolution of misregulated genes may be that these genes are non-essential and thus are subject to relaxed selective constraint. The paucity of genes with lethal mutant phenotypes among those misregulated would appear to support this notion (see below). Application of population genetic analyses that are more sensitive to detecting weaker signatures of positive selection in closely related species (i.e. those incorporating polymorphism information between and within species; Eyre-Walker 2006) will be required to determine whether directional selection or relaxed constraint is the more likely explanation for the patterns observed.

Several studies have shown that genes that are considered essential (i.e. have lethal mutant phenotypes) are more conserved over evolutionary time than those that are considered dispensable (Torgerson and Singh 2006; Hahn et al. 2006 ; He and Zhang 2006). Our study demonstrated that not only are genes with a known lethal mutant phenotype evolving more slowly at the sequence level, but that they are also less likely to be under-expressed relative to parental species in interspecific hybrids. This suggests that the phenomenon of gene misregulation in interspecific hybrids is occurring predominantly among genes whose mutants do not display lethal phenotypes, possibly due to the effect of strong purifying selection acting on genes with severely deleterious mutant phenotypes.

Several mechanisms have been proposed in order to account for the misregulation of genes in interspecific hybrids (for review see Ortiz-Barrientos et al. 2006). Such mechanisms include the divergent coevolution of transcription factors and their binding sites between species (Johnson and Porter 2000) such that they fail to complement in the hybrid background. Other mechanisms involve species-specific loss of regulatory pathway elements (such that the pathways no longer compliment each other in the hybrids) and divergent evolution of alternatively spliced transcripts and other forms of post-translational modification (Ortiz-Barrientos et al 2006).

All such mechanisms share a common feature in that they predict that the most rapidly evolving (and thus divergent) genes will be those that are most likely to be subject to misregulation in inter-specific hybrids. Simulations of these conditions have found that the rate of accumulation of incompatibilities between populations increases when the

affected loci are under directional selection (Johnson and Porter 2000; 2007). These predictions are partially validated by our observation that under-expressed misregulated genes evolve more rapidly than non-misregulated genes (Figure 3.1; Supplementary Table 2), as well as the observation that male-biased genes (MBGs) are over- represented among them.

Unfortunately, these mechanisms do not seem to account for our observation that over-expressed misregulated genes evolve less rapidly than non-misregulated genes (Figure 3.1; Supplementary Table 1 and 2). A closer inspection of the predicted functions of these genes using FATIGO (Al-Shahrour et al 2004) reveals that they are enriched in proteins involved in translation (more specifically ribosomal proteins; $p = 5.88\times10^{-5}$) relative to under-expressed misregulated genes. Ribosomal production may be upregulated in hybrids in order to maintain viability under the burden of reduced expression of many genes, though such an effect would only be proximally related to hybrid misregulation rather than a direct effect of hybrid incompatibility. Female-biased genes (FBGs) are also over-represented among over-expressed misregulated genes (Haerty and Singh 2006). Such genes have been shown to evolve less rapidly than MBGs or genes that do not show sex-bias (Meiklejohn et al. 2003). Proper male sex-determination and differentiation in Drosophilids requires the activation of male-determining genes as well as the concomitant repression of female-determining genes (for review see Schütt and Nöthiger 2000). The overwhelming misregulation of MBGs in hybrids could produce a lack of proper repression of FBGs, leading to an overall pattern of over-expression among these conserved transcripts.

While such mechanisms can account for why a portion of the most conserved misregulated genes tend to be over-expressed, it is quite probable that additional mechanisms also play important roles in the phenomenon. For instance, 75 of the over-expressed misregulated genes are classified as MBGs (Supplementary Table 1). Analyzed as a group, these genes are still evolving less rapidly than non-misregulated genes in both d_N and d_N/d_S (Kruskal-Wallis test, $p < 2.2\times10^{-16}$ and $p = 2\times10^{-4}$ respectively), while no difference is observed for the d_S (Kruskal-Wallis test, $p= 0.0668$) indicating that additional evolutionary processes are responsible for the coupling of sequence conservation and over-expression in inter-specific hybrids. Additional functional studies leading to a better understanding of the mechanisms of gene regulation may be required to identify these processes.

Correlation between parental expression and sequence divergence

As previous studies have shown, we also observed a significantly positive correlation between expression difference between parental species and the d_N or d_N/d_S ratio (Castillo-Davis et al. 2004; Nuzhdin et al. 2004; Lemos et al. 2005). However, these results are in contrast with Good et al.'s (2006) finding that genes with divergent expression between species do not have a significantly higher d_N/d_S than genes with

similar levels of expression. Such a discrepancy may be due to the larger number of genes used in the present study as well as that our analysis is restricted to genes expressed within the testis, which are known to present a higher variation in sequence and expression differences between species (Meiklejohn 2003; Ranz et al. 2003).

In conclusion we find that under-expressed misregulated genes in interspecific sterile hybrids are evolving more rapidly at the coding level than genes that are not misregulated or over-expressed misregulated genes and that gene expression differences between hybrids and parental species are significantly correlated with coding sequence divergence. Our observation that misregulated genes, specifically those that are under-expressed, show an under-representation of genes with known lethal mutant phenotypes would suggest that similar selective pressures are acting to maintain expression levels as well as to minimize sequence divergence of essential genes between species. The phenomenon of gene misregulation in hybrids appears to involve both a more rapid evolution of coding sequences coupled with an enrichment of male-biased genes among those misregulated (e.g. Michalak and Noor 2003; Haerty and Singh 2006). Previous studies have shown that male-biased genes are evolving more rapidly, probably due to sexual selection (Swanson and Vacquier 2002; Singh and Kulathinal 2000; Jagadeeshan and Singh 2005); therefore, this suggests that sexual selection may be the driving force behind the rapid divergence of the mostly male-biased, misregulated genes observed in this study.

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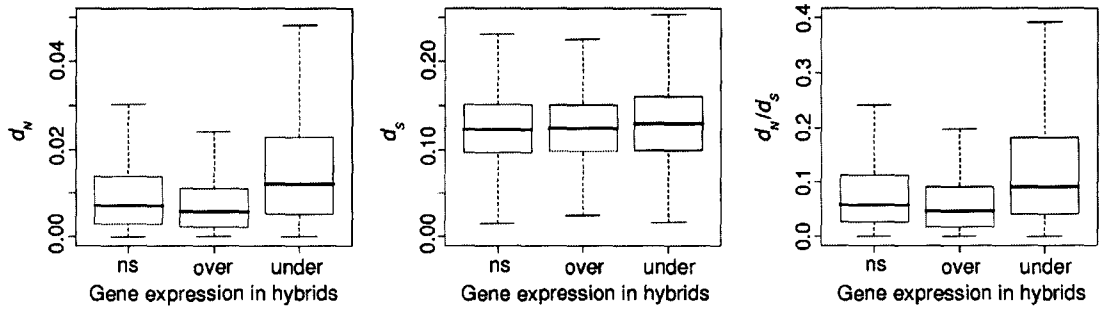

Figure 3.1: Comparison of d_N, d_S and d_N/d_S between non-misregulated genes and misregulated genes in hybrids. Misregulated genes are divided into those under-expressed and those over-expressed relative to parents.

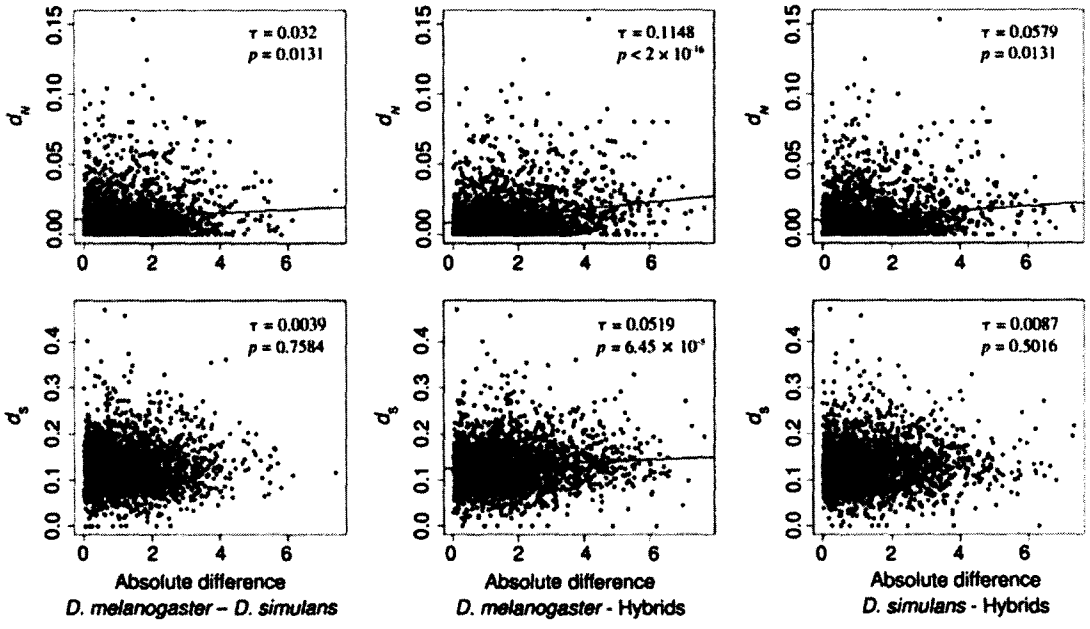

Figure 3.2: Relationship between sequence divergence (d_N, d_S) and absolute gene expression difference between *D. melanogaster* and *D. simulans* species and between parental species and hybrids. Kendall rank sum coefficients of correlation (τ) and p-values are shown in each frame. The correlation between expression difference and dN/dS is not shown, however the values are $\tau = 0.1104$ and $p = 1.44 \times 10\text{-}14$, $\tau = 0.0561$ and $p = 1.77 \times 10\text{-}5$, $\tau = 0.0269$ and $p = 0.0389$ for the correlations with expression difference between *D. melanogaster*/hybrid, *D. simulans*/hybrid and *D. melanogaster*/*D. simulans* respectively.

Table 3.1: Comparison of average (± standard deviation) evolutionary rates for genes showing similar levels of expression between *D. melanogaster* and *D. simulans*.

	Expression in hybrids		
	non-misregulated	misregulated	
		over	under
N	1112	245	484
d_N	0.0106 (±0.0123)	0.0087 (±0.0080)	0.0176 (±0.0165)
d_S	0.1286 (±0.0478)	0.1293 (±0.0477)	0.1316 (±0.0510)
d_N/d_S	0.09 (±0.1233)	0.0672 (±0.0729)	0.1312 (±0.1382)

Table 3.2: Relationship between expression difference and sequence divergence for genes showing similar levels of expression between *D. melanogaster* and *D. simulans*.

		D. melanogaster/ hybrids		*D. simulans* / hybrids	
		over	under	over	under
d_N	τ[1]	-0.0119	0.1188	-0.0408	0.086
	P-value	0.7678	2.89×10^{-6}	0.3115	7.063×10^{-3}
d_S	τ	0.0074	0.0546	0.0507	0.0497
	P-value	0.853	0.0312	0.2061	0.0498
d_N/d_S	τ	-0.0035	0.1030	-0.0221	0.0750
	P-value	0.9312	5×10^{-5}	0.5845	0.0032

[1] Kendall rank sum coefficient of correlation

CHAPTER 4

Ontogeny and Phylogeny: Molecular Signatures of Selection, Constraint, and Temporal Pleiotropy in the Development of *Drosophila*

This chapter has been published in BMC Biology:

Artieri Carlo G., Wilfried Haerty, and Rama S. Singh. 2009. Ontogeny and Phylogeny: molecular signatures of selection, constraint, and temporal pleiotropy in the development of Drosophila. *BMC Biology* 21, no. 7 (July): 42.

In this article, we used stage-specific *Drosophila melanogaster* EST libraries in order to classify genes into developmental stages and test whether genes expressed early in development are more conserved between species at the coding sequence level as may be predicted from Von Baer's classical observations (see § 1.3.2). Furthermore, we were also able to test predictions made by the developmental constraint hypothesis and Darwin's selection opportunity hypothesis, which both seek to explain Von Baer's laws from a molecular perspective. W. Haerty and I outlined the study. I collected the data and performed the computational and statistical analyses. I also drafted the manuscript and all three authors assisted in final editing and revisions. Please note that supplementary materials can be found online at http://www.biomedcentral.com/1741-7007/7/42/.

4.1 Abstract

BACKGROUND: Karl Ernst Von Baer noted that species tend to show greater morphological divergence in later stages of development when compared to earlier stages. Darwin originally interpreted these observations via a selectionist framework, suggesting that divergence should be greatest during ontogenic stages in which organisms experienced varying 'conditions of existence' and opportunity for differential selection. Modern hypotheses have focused on the notion that genes and structures involved in early development will be under stronger purifying selection due to the deleterious pleiotropic effects of mutations propagating over the course of ontogeny, also known as the developmental constraint hypothesis.

RESULTS: Using developmental stage-specific EST libraries, we tested the two hypotheses by comparing the rates of evolution of 7,180 genes obtained from 6 species of the *Drosophila melanogaster* group with respect to ontogeny, and sex and reproduction related functions in gonadal tissues. Supporting morphological observations, we find evidence of a pattern of increasing mean evolutionary rate in genes that are expressed in subsequent stages of development. Furthermore, supporting expectations that early expressed genes are constrained in divergence, we find that embryo stage genes are involved in a higher mean number of interactions as compared to later stages. We note that the accelerated divergence of genes in the adult stage is explained by those expressed specifically in the male gonads, whose divergence is driven by positive selection. In addition, accelerated gonadal gene divergence occurs only in the adult stage, suggesting that the effects of selection are observed primarily at the stages during which they are expected occur. Finally, we also find a significant correlation between temporal specificity of gene expression and evolutionary rate, supporting expectations that genes with ubiquitous expression are under stronger constraint.

CONCLUSIONS: Taken together, these results support both the developmental constraint hypothesis limiting the divergence of early expressed developmentally important genes, leading to a gradient of divergence rates over ontogeny (embryonic < larval/pupal < adult), as well as Darwin's 'selection opportunity' hypothesis leading to increased divergence in adults, particularly in the case of reproductive tissues. We suggest that a constraint early/opportunity late model best explains divergence over ontogeny.

4.2 Background

For over a century, developmental biologists have noted an ontogenic pattern among evolutionary relationships: earlier developmental stages are morphologically more similar across species than later stages, which is also known as Von Baer's third law (Von Baer 1828; Gould 1977; Reidl 1978; Richardson et al. 1998). While more recent studies in vertebrates have determined that the very earliest stages of ontogeny (e.g., gastrulation)

may be subject to substantial variation even among closely related species, upon reaching the tailbud stage, embryos begin to share more similarity in appearance, which gradually declines with subsequent development (Raff 1996). This 'hourglass' model of developmental similarity among vertebrates suggests that while certain stages of development undergo substantial change over evolutionary time, there exists a significant conservation of the mechanisms underlying development across vertebrates (Seidle 1960; Sander 1983; Hall 1997; Galis and Metz 2001). Darwin originally interpreted Von Baer's observations via a selectionist framework (Darwin 1871; 1872). He suggested that divergence should be greatest during ontogenic stages in which organisms experienced the most varying 'conditions of existence' and, as a result, occasioned opportunity for differential selection (Gould 1977). Embryos of varied species are therefore more similar than adults due to exposure to very similar fetal environments. Furthermore, he noted that derived features rarely appeared in an organism before the stage when they were used, indicating that the effect of selection was also specific to the stage where selection pressure actually occurred. This observation was important to his overall hypothesis, as selection pressures occurring during one stage that selected for traits expressed in other stages would be inconsistent with Von Baer's observations. Using secondary sexual traits as a primary example, Darwin compiled a large number of observations indicating that male-specific structures known to be highly divergent even among closely related species rarely developed until reproductive maturity was reached (Darwin 1871; Eberhard 1985).

Modern interpretations of Von Baer's third law have focused on another, non-mutually exclusive mechanism: genes implicated in early aspects of development are more likely to regulate a large number of downstream effectors via hierarchical regulatory cascades, and are thus more evolutionarily constrained due to the large deleterious pleiotropic effects of mutations. This is known as the developmental constraint hypothesis (Reidl 1978; Arthur 1988; Cutter and Ward 2005). The complex hierarchical nature of gene regulatory networks has become a focus of major interest in the field of organismal development (Davidson et al. 2003; Wittkopp 2007) with special attention being paid in particular to those network modules critical to early development, and conserved over broad evolutionary distances (Davidson and Erwin 2006). For instance, the well-known homeotic genes, involved in establishing the anterior/posterior axis in the early development of most metazoans, provide a striking example of highly conserved genes whose mutations are known to have extensive pleiotropic consequences (Lewis 1978; Lutz et al. 1996; Lemons and McGinnis 2006). These transcription factors are also known to act as master regulatory switches in cascades involved in regulating the proper expression of many downstream, developmentally important effectors (Carroll 1995). Another example is the gene regulatory feedback loop required for endoderm specification in echinoderms, which encodes several transcription factors whose inactivation has catastrophic effects on the entire body plan (Davidson and Erwin 2006; Hinman et al. 2003). These instances highlight the strength of purifying selection acting on specific genes known to be involved in complex developmental regulatory networks;

however a more recent interest has concerned the broader evolutionary patterns of the genome with respect to ontogeny.

The evolutionary dynamics of genes expressed over the course of development have recently been examined at the genomic level in the case of flies and nematodes using microarray based information about the developmental timing of gene expression (Castillo-Davis and Hartl 2002; Cutter and Ward 2005; Davis et al. 2005). Castillo-Davis and Hartl (2002) used previously published, developmental stage–specific microarray data (Hill et al. 2000) in order to compare the rates of coding sequence divergence of a relatively small number of genes (224) between *Caenorhabditis elegans* and *C. briggsae* (20 – 120 million years diverged [MYD]). Genes in their dataset were classified either as 'nonmodulated' (i.e., invariant in expression level over development), early-expressed (i.e., embryonic), or late-expressed (i.e., larval and adult) based on the developmental stage at which their peak level of expression occurred. The authors found no significant difference in the rates of protein evolution among the three categories, though the early-expressed genes showed a higher rate of synonymous substitution as well as a lower codon usage bias (CUB) than late-expressed genes. The analysis of the same two species was subsequently refined by Cutter and Ward (2005) using a larger dataset of 7,281 genes and a larger source of developmental expression data (Baugh et al. 2003; Reinke et al. 2004). Their results support some theoretical predictions of both the developmental constraint as well as Darwin's 'selection opportunity' hypothesis: When genes were classified based on the stage at which their peak expression level occurred, adult genes were found to be evolving more rapidly than those in the earlier, larval stage. Expression level in the larval stage, relative to the adult, was also found to be negatively correlated with sequence divergence, while the opposite was observed for expression in adults. However, the authors noted no unidirectional trend in evolutionary rates in genes expressed over the course of embryogenesis, as would be predicted by the developmental constraint hypothesis, leading them to suggest that constraint may not explain the evolutionary rates of proteins expressed during embryonic development in these species. Furthermore, when examining the tissue specificity of genes expressed in adult nematodes, the authors found that the majority, though not all, of the acceleration in evolutionary rate observed in this stage was explained by genes expressed primarily in the male gametes, providing evidence of a significant effect of sexual selection, presumably acting through sperm competition between males and hermaphrodites or antagonistic co-evolution between genes expressed in sperm and oocytes (Cutter and Ward 2005).

Davis et al. (2005) used the results of a microarray study of the expression levels of 4,028 genes over the course of *Drosophila melanogaster* ontogeny (Arbeitman et al. 2002) and examined their rates of sequence divergence between *D. melanogaster* and *D. pseudoobscura* (25 – 55 MYD). They noted that gene expression level in the late embryo relative to later stages was negatively correlated with sequence divergence, while the opposite was observed in the case of adult males. However, the authors noted no significant correlation between expression levels and sequence divergence for the many

of the sampled developmental stages. Unfortunately the species pairs used in both of these studies were quite distantly diverged and thus interpretation of these data is limited due to the saturation of synonymous site divergence (d_S), which largely prevents investigation of questions regarding evidence of selection (Graur and Li 2002; Musters et al. 2006). Furthermore, comparisons at such evolutionary distances allow the possibility that expression patterns (e.g., time of expression, sex-bias, etc.) have diverged between species, questioning whether similar selective pressures are acting along both lineages at the level of individual genes (Zhang et al. 2007).

Holometabolous insects such as *Drosophila* provide an excellent model for studying gene evolution over ontogeny as they pass through four separate, unambiguous developmental stages (embryo, larva, pupa, and adult). A large body of information about the evolutionary dynamics of the genomes of drosophilids has accumulated, aided significantly by the recent release and analysis of the complete genomes of 12 *Drosophila* species (Drosophila 12 Genomes Consortium 2007). However, the relationship between development and genomic evolution remains largely unexplored. Here we analyze a larger dataset than was previously available, using information generated from publicly available developmental stage-specific EST libraries to assign genes to specific developmental stages and determine their evolutionary patterns within the *D. melanogaster* group, allowing more reliable estimates of divergence parameters as well as reducing the caveats associated with comparing distantly related species (Davis et al. 2005). We report a gradient of increasing mean evolutionary rate in genes expressed in subsequent stages of fly development, culminating in exaggerated gene sequence divergence specifically in adult males. When comparing genes expressed specifically in the gonads of embryos to adults, we find that the increased rate of divergence observed in adults is explained entirely by those genes expressed in the testis. No such pattern of accelerated gene divergence is observed in the embryonic gonads supporting Darwin's expectations that selection pressures should act predominantly in the stage where opportunity for selection occurs (Darwin 1871). Finally when classifying genes into specific developmental stages using a series of increasing stage-specificity thresholds, we find a significant correlation between specificity of temporal stage of expression and evolutionary rate. We also reanalyze the dataset used by Davis et al. (2005) using our methods in order to refine their estimates of divergence and test the generality of their results (see Appendix § 4.11). Taken together, our results support both developmental constraint acting to limit the divergence of early expressed, developmentally important genes (Raff 1996; Galis and Metz 2001), as well as the notion that accelerated divergence in adults is primarily due to increased selection pressures occurring during this stage.

4.3 Results

Analysis of the EST library based developmental profile

We obtained developmental stage specific information for 7,180 genes found in the 6 species of the *D. melanogaster* group (~17 MYD) (Lachaise et al. 1988) in the *Drosophila* 12 Genomes Consortium (2007) dataset from UniGene (Pontius et al. 2003) (see Methods), representing a ~2.5 fold increase in size over the *Drosophila* developmental time-course dataset used by Davis et al. (2005). We were unable to obtain separate libraries representing the larval and pupal stages, therefore we pooled all available EST libraries into three developmental stages based on the stage during which they were generated: embryonic, larval/pupal, and adult. Genes were classified into developmental stages based on the stage during which they show their highest proportion of representation in the EST libraries, under the assumption that this represents a biologically reasonable proxy of when the majority of a gene's function(s) occur. However, given that such a method of classification may be subject to complications arising from normal within individual variation in gene expression levels, we re-classified genes using increasing stage-specificity thresholds (see Material and Methods; Table 4.1; Supplementary Table 1).

As a test of our assumption that a gene's highest stage of expression is also the stage during which the majority of its functions occur, we performed pairwise comparisons of the lists of genes classified at each stage for each specificity threshold using FatiGO (Al-Sharour et al. 2004, 2007) (Supplementary Table 2). We found that certain 'Biological Process' Gene Ontology (GO) terms associated with temporal-specific functions were consistently over-represented among genes classified into the stage(s) during which such functions were expected to occur. For instance, in the embryogenic *versus* adult comparison, terms associated with development and regulation (e.g., 'regulation of biological process' [GO:0050789], and 'multicellular organismal development' [GO:0007275]) were consistently over-represented among genes classified as embryonic, while terms associated with detection and response to external stimuli were over-represented among genes classified as adult (e.g., 'detection of stimulus' [GO:0051606], and 'response to abiotic stimulus' [GO:0009628]). Similar trends were observed in the comparison between the combined larval and pupal stages *versus* the adult stage, where for example, the term 'post-embryonic development' (GO:0009791) was over-represented among larval/pupal genes, as expected. In the comparison between the embryonic *versus* larval/pupal stages terms associated with regulation (e.g., 'regulation of biological process' [GO:0050789]) tend to be over-represented among embryonic genes while those associated with energy metabolism (e.g., 'generation of precursor metabolites and energy' [GO:0006091] and 'carbohydrate metabolic process' [GO:0005975]) tend to be over-represented in the larval/pupal stage, as may be expected given the large amount of organismal growth occurring during the larval stage (Vicario et al. 2008). Curiously, the term 'sexual reproduction' (GO:0019953) is consistently over-

represented among genes classified as being specific to the embryonic and larval/pupal stages as compared to the adult stage (Supplementary Table 2). These genes may be associated with organogenesis of sexual organs, which occurs prior to adulthood, or with spermatogenesis, which begins in the 3rd instar larval stage (Hartenstein 1993). However, in general, terms were over-represented in pairwise comparisons in the expected direction, providing support to our assumption of an association between expression level and temporal function.

We found that adult stage genes are evolving more rapidly than earlier stages in d_N, d_S, and d_N/d_S at most specificity thresholds (Kruskal-Wallis rank sum test $p < 0.01$; Bonferroni correction was applied to all pairwise comparisons) (Figure 4.1; Supplementary Table 2). Moreover, at all specificity thresholds, genes classified into the larval/pupal stage experience a higher d_N/d_S than those of the embryonic stage ($p < 0.05$). To our knowledge, this represents the first empirical evidence of a gradient in evolutionary rates spanning the whole of *Drosophila* ontogeny, wherein genes represented at their highest level in the adult are evolving more rapidly than those in the pooled larval and pupal stages, and both are evolving more rapidly than those in the embryonic stage (i.e., embryonic < larval/pupal < adult). Under most specificity thresholds, the d_N of larval/pupal genes was also significantly greater than those of embryo genes; however, the d_S of larval/pupal genes was significantly lower than that of embryonic genes, such that the relationship among stages in terms of the d_S was larval/pupal < embryonic < adult (Supplementary Table 3). Previous studies have demonstrated a positive relationship between tissue specificity of expression and evolutionary rate, presumably due to selection against the deleterious pleiotropic effect of mutation restricting the divergence of broadly expressed genes (Khaitovich et al. 2005; Haerty et al. 2007; Larracuente et al. 2008). We sought to test for a similar relationship between temporal specificity of gene expression (i.e., stage specificity) and the rate of evolution by comparing the mean d_N/d_S between our specificity thresholds at each of our three developmental stage classifications (Figure 4.1). We performed Bonferroni corrected, pairwise permuted Kruskal-Wallis tests between the distributions of divergence parameters at each of the specificity thresholds within each stage (Supplementary Table 4), and found that there is a clear relationship between the stage specificity of representation in EST libraries and the mean evolutionary rate of genes at that stage: for most comparisons, the more specific a gene's representation at a particular stage, the higher its rate of divergence ($p < 0.05$) (Figure 4.1). The large confidence intervals associated with the larval/pupal stage are likely due to a reduced number of genes classified as specific to this stage, especially in the case of the higher specificity thresholds (Table 4.1). Similar results are seen in the case of d_N, however, in the case of d_S there was no significant difference between specificity thresholds (Supplementary File 2), with the exception of the adult stage, where the highest specificity thresholds have a significantly higher d_S than low specificity thresholds (e.g., genes showing greater adult stage specificity tend to have a higher d_S) (Supplementary Table 4).

The selection opportunity hypothesis (Darwin 1871) predicts not only that the average rate of change must increase over developmental stages, but that the proportion of genes showing evidence of positive selection should increase with subsequent developmental stages (Good and Nachman 2005). We tested this prediction by performing pairwise comparisons of the proportion of genes showing significant evidence of positive selection using the comparison between models 7 and 8 in Phylogenetic Analysis by Maximum Likelihood (PAML) (Yang and Neilsen 2000) according to the *Drosophila* 12 Genomes Consortium (2007) data, at each stage and for each specificity threshold (Supplementary Table 1). After applying a Bonferroni correction for multiple tests, we found no significant differences in the proportion of genes showing evidence of positive selection in any pairwise comparisons between stages (Supplementary Table 5).

Stage specificity of selection pressure

A key postulate of the selection opportunity hypothesis (Darwin 1871) is that the effects of late-stage acting selective pressures primarily affect features specific to the stage at which they occur. As a test of this hypothesis, we sought to compare the effect of expression of genes within gonads relative to those expressed in the rest of the body at the two stages in which we had tissue-specific EST library representation information: embryo and adult. Genes were separately classified into either 4 different stage/tissue categories (embryonic general, embryonic gonads, adult general, and adult gonads) or 5 tissue categories (wherein the adult gonad library is separated into adult ovary or adult testis) (see Methods; Table 4.1; Supplementary Table 1). It should be noted that the 'embryonic general' class was generated from whole-body tissue (including the gonads), and also that in the generation of the embryonic gonad EST libraries individuals were not sexed, and thus the ESTs reflect undifferentiated gonads pooled from both sexes (Shigenobu et al. 2006).

We found that genes classified as being expressed in combined adult gonads are evolving significantly more rapidly than all other stages in d_N, d_S, and d_N/d_S at all specificity thresholds ($p < 0.01$), with sole exception of the comparison between the d_S of adult and embryonic gonads, which is non-significant (Figure 4.2; Supplementary File 3; Supplementary Table 6). When adult gonads are separated into either 'adult ovary' or 'adult testis', we find that only genes expressed in the testes show an accelerated mean rate of evolution (d_N and d_N/d_S) relative to other stages. Under those specificity thresholds where a significant difference in evolutionary rate was found between adult ovaries and other tissues, the mean rate of evolution of genes expressed in the ovaries was significantly lower than the other tissues ($p < 0.05$; Figure 2; Supplementary File 3; Supplementary Table 6). Contrary to what is observed in the adult, genes expressed in the embryonic gonads are evolving more slowly than non-gonadal tissues (d_N and d_N/d_S; $p < 0.05$). We found no consistent significant differences in the rate of evolution between genes expressed in non-gonadal adult or embryonic tissue, supporting the results of

previous studies indicating that gonadal expression plays a large role in explaining evolutionary rate differences in the adult stage (Cutter and Ward 2005; Davis et al. 2005).

As in the case of genes classified into specific stages, we performed pairwise comparisons of the proportion of genes showing evidence of positive selection for each tissue/stage and for each specificity threshold. Again, no comparisons were statistically significant after Bonferroni correction, with the sole exception that genes classified as unique to the adult testis have a significantly higher proportion of genes showing evidence of positive selection than genes classified as unique to the adult general category (χ^2 value = 8.76 , df = 1, p = 0.0308) (Supplementary Table 5).

Gene interaction profiles during development

The development constraint hypothesis is predicated on the notion that development is coordinated by hierarchical genetic networks (Wright 1990; Davidson et al. 2003; Wittkopp 2007) and therefore features of early development are more likely to be constrained by selection against the deleterious pleiotropic effects of mutations (Reidl 1977; Raff 1996; Davidson and Erwin 2006). A logical prediction of such theory is that genes involved in earlier stages of development should represent more central regulatory nodes and, on average, be involved in more interactions as a consequence. Using the BioGRID database (Stark et al. 2006) we obtained the total number of interactions associated with each gene from the EST-based dataset for which interaction information was available, resulting in a total of 4,422 genes involved in 34,462 interactions (Supplementary Table 7). We found a significantly higher mean number of interactions per gene for genes specific to the embryonic stage as compared to the larval/pupal stage at no specificity threshold and a greater than two-fold proportional representation threshold (Kruskal-Wallis permuted rank sum test, p = 0.0162 and 0.0003 respectively; Table 4.2; Supplementary Table 7; Supplementary Table 8). The mean number of interactions was also higher for genes specific to the embryo as compared to the adult stage at most specificity thresholds ($p < 0.05$; Supplementary Table 8). All other comparisons, including those between the larva/pupal and adult stages were non-significant. In order to minimize the potential effect of stage-specific ascertainment bias in the BioGRID database's genetic interaction data (i.e., a greater proportion of genetic interaction experiments are likely performed during embryogenesis), we also performed the same analysis using only BioGRID's data on direct protein-protein interactions (yeast two-hybrid; 4092 genes involved in 23,712 interactions; Supplementary Table 8). Our results remained qualitatively unchanged when using all yeast two-hybrid interaction data, however the majority of statistically significant comparisons disappeared when we limited our analysis only to the 'high-confidence interactions' as defined by Giot et al. (2003) (2,736 genes involved in 5,589 interactions, Supplementary Table 8), though the embryonic stage continues to show a higher mean number of interactions as compared to

the larval/pupal stage at no specificity threshold and a greater than two-fold proportional representation threshold ($p = 0.0165$ and 0.0471, respectively).

When comparing the average number of interactions per gene between gonadal and non-gonadal tissues in the adult and embryonic stages, we observed significantly fewer interactions in both the adult non-gonad and adult gonad categories as compared to the embryonic general category at no specificity threshold and a greater than two-fold proportion of representation threshold ($p < 0.05$). The embryonic gonad category showed a significantly higher mean number of interactions than both adult general and adult gonad categories only when no specificity threshold was used in classification ($p < 0.05$ after Bonferroni correction). No other pairwise comparisons of mean number of interactions per gene were statistically significantly different, including both within-stage comparisons of gonadal to non-gonadal tissue. When adult gonads were separated into either ovary or testis-specific genes, only genes classified as testis specific had significantly fewer mean interactions ($p < 0.05$ at no specificity threshold and a greater than two-fold proportion of representation threshold). As was the case above, we reanalyzed the data using only direct protein-protein interactions and again, results were qualitatively similar, though no pairwise comparison was statistically significant after Bonferroni correction when adult ovaries and testes were classified separately (with the sole exception of the embryo general category which shows a significantly higher mean number of interactions than the adult general category using no specificity threshold, $p = 0.0180$). Also similarly, limiting our analysis to 'high-confidence' interactions resulted in most of the significant comparisons to becoming non-significant, likely owing to the smaller total number of interactions as compared to the total dataset (Supplementary Table 8).

Previous studies have demonstrated a significant negative correlation between the total number of interactions in which genes were involved and their rate of evolution (Fraser 2005; Lemos et al. 2005). Given our observation that increased stage specificity was positively correlated with evolutionary rate, we tested for a significant correlation between the number of stages in which a gene was represented and its number of interactions. We found a significant positive correlation between the number of stages in which genes are represented and the number of interactions in which they are involved (Kendall rank sum correlation test $\tau = 0.0848$, $p = 4.501 \times 10^{-12}$).

4.4 Discussion

Our study provides molecular confirmation of two different but non-mutually exclusive hypotheses seeking to explain Von Baer's 'Third Law', noting that morphological similarity among organisms tends to decrease over ontogeny (Von Baer 1828). Our findings consist of (1) evidence for stronger purifying selection during embryonic development as predicted by the modern developmental constraint hypothesis

(Reidl 1978; Raff 1996), (2) evidence for selection-driven accelerated divergence of genes in the adult stage, exemplified by those expressed in males, as predicted by Darwin (1871), and (3) the existence of a temporal pleiotropy restricting the divergence of genes that are broadly expressed over the course of development.

Expression patterns across the *Drosophila* phylogeny

All developmental and spatial representation of gene expression information in our study is based on data collected in *D. melanogaster*, therefore an underlying assumption is made that developmental and spatial expression patterns, or more specifically that the stage/tissue of highest expression level, do not vary significantly among species of the *D. melanogaster* subgroup. While several studies have shown considerable variation in expression levels between species at the adult stage (Ranz et al. 2003; Meiklejohn et al. 2003), to our knowledge, there are no studies that have directly compared expression levels between species over development on a large scale. A study conducted by Rifkin et al. (2003) found that approximately 17% of genes surveyed (2,193/12,866) had significant differences between species in the degree to which genes in expression pattern changed during the onset of metamorphosis in *D. melanogaster*, *D. simulans*, and *D. yakuba*. However, it is unclear if such changes imply that the stage of highest level of expression changes between species. Regardless, if patterns of expression varied considerably between the species used in our study, we would expect this to add noise to the evolutionary signals we observed rather than produce systematic biases in our dataset.

Divergence patterns over development

The results of our analysis indicate that sequence follows the pattern observed in morphology over the course of development: we observed a positive gradient in the rates of divergence (d_N and d_N/d_S) in subsequent stages of ontogeny (Figure 1; Supplementary Figure 4.1). However, in the case of the synonymous rate of substitution, d_S is highest in adults and lowest in the larval/pupal stage (i.e., larval/pupal < embryonic < adult) (Supplementary Figure 2). These observations are consistent with either a) systematic variation in the level of codon-usage bias between developmental stages, or b) a systematic difference in the rate of mutation between stages of development. A recent study performed by Vicario et al. (2008) confirmed that CUB varies significantly among developmental stages when estimated in both *D. melanogaster* and *D. pseudoobscura*. Furthermore, the pattern of variation in CUB that they observed (adult < embryonic < larval) mirrors the rate of synonymous substitution measured at each stage in our study, consistent with CUB being responsible for the patterns of variation in d_S that we observe (i.e., high CUB reduces d_S by selecting against substitutions generating non-optimal codons) (Akashi 2001). A similar analysis of the Codon Adaptation Index (Sharp and Li 1987) using codonW (http://codonw.sourceforge.net) on our dataset agreed with Vicario

et al.'s (2008) results (data not shown). While it is not possible to rule out the hypothesis of different mutation rates affecting genes expressed in different stages of ontogeny, the non-concordance between the patterns observed in the synonymous and non-synonymous rates of substitution, d_S and d_N, indicates that differential mutation rate alone is insufficient to explain the positive gradient of divergence in d_N and d_N/d_S observed over ontogeny. However, a gradient in these divergence rates over development is predicted by both the developmental constraint and selection opportunity hypotheses and thus evidence supporting either or both will be considered below.

Embryonic developmental constraint

Supporting the developmental constraint hypothesis, we observed an increased mean number of interactions per gene among genes showing their highest level of expression in the embryonic stage when compared to those specific to other stages (Table 4.2). This is consonant with the notion that the products of genes expressed in this stage are involved in a greater number of highly connected regulatory networks, and are thus constrained in their divergence due to the cascading effects of deleterious mutations (Davidson et al. 2003; Wittkopp 2007). We observed that genes classified as specific to the embryonic gonadal category were involved in significantly more interactions than those specific to the adult gonads, suggesting that lack of pleiotropy-mediated constraint may play some role in explaining the tolerance for evolutionary divergence of adult gonad specific genes when compared to those of other tissues and stages - particularly in the case of the testis (Supplementary Table 6).

A potential caveat to such analysis could occur if the majority of interaction studies in *Drosophila* were performed with the intention of identifying interactions in the embryo, thus biasing the data in favor of a greater number of embryo-specific gene interactors. However, when we limited our analysis to interactions derived from yeast two-hybrid experiments using gene predictions from the whole *Drosophila melanogaster* genome (Giot et al. 2003; Stanyon et al. 2004), our results remained qualitatively unchanged, suggesting that our dataset is not significantly biased towards any specific stage. It should be noted that the yeast two-hybrid technique is known to generate a large number of false-positive predictions of protein-protein interactions (reviewed in Hart et al. 2006). However, in order for such false-positives to have a significant effect in biasing our data, it would require that the whole genome yeast two-hybrid studies from which the interaction data are derived (Giot et al. 2003; Stanyon et al. 2004) preferentially produce false positives among genes expressed at their highest level in the embryonic stage. A large number of interactions in BioGRID's database are not derived from yeast two-hybrid studies, and limiting our analysis to these studies supports the results observed from the analysis of the entire data set (data not shown). However, it is likely that interactions derived from these genetic studies are biased towards experiments conducted during embryogenesis, and thus such observations should be interpreted with caution.

Noting that very early ontogenic processes such as gastrulation can show considerable divergence among closely related species, Raff (1996) suggested that developmental constraint may imperfectly reflect the sequence of organismal ontogeny, but rather that the constraining effects of pleiotropy should be highest during those developmental stages showing the least amount of modularity, or disassociation, between regulatory pathways. It is possible that, given the large-scale morphogenesis that occurs during both embryogenesis and metamorphosis in *Drosophila*, more genes expressed during the embryonic and pupal stages occur in highly interconnected regulatory networks and thus are constrained by greater pleiotropy than those specific to the larval and adult stages. However, our analysis of the mean number of interactions of genes classified into the pooled larval and pupal stages found no significant difference when compared to genes classified into the adult stage (Table 4.2; Supplementary Table 8). While this may be an effect of larval stage genes obscuring the signal of a greater number of interactions in the metamorphosis stage, this seems unlikely as under the strict predictions of the developmental constraint hypothesis, larval genes should be, on average, more conserved than those of the subsequent metamorphosis stage and therefore possibly involved in more interactions. Unfortunately, separate larval and pupal derived EST libraries will be required to answer such concerns. It should be noted that Arbeitman et al. (2002) observed that the transcriptomes of the embryonic and pupal stages are more similar to one another than either is to the larval or adult, suggesting that many genes classified as embryonic specific may have important functions in metamorphosis.

Selection opportunity and adult divergence

Unlike the developmental constraint hypothesis, which predicts that the gradient in divergence rates observed over ontogeny is produced by relaxed selective constraint occurring on genes expressed in later stages, Darwin's (1871) selection opportunity hypothesis argues that this gradient is driven by positive selection. Unfortunately, an increase in d_N and d_N/d_S over development, as we observed, is consistent with both positive selection and relaxed selective constraint. However, as part of the predictions of the selection opportunity hypothesis, we should also observe an increase in the proportion of positively selected genes in later stages of development (Good and Nachman 2005). When examining the proportion of genes showing evidence of positive selection among our 3 developmental stages, the differences between stages were not statistically significant (Supplementary Table 5). It should be noted however, that the number of genes in our dataset showing significant evidence of positive selection was quite small (359 out of 7,180 genes classified under no specificity threshold) and may represent too limited a dataset from which to draw statistically meaningful conclusions. While this may suggest that our results do not support Darwin's hypothesis, it is interesting that our study of both EST and microarray based datasets noted that the accelerated rate of evolution observed in the adult stage is explained by the rapid evolution of male-biased genes – and more specifically, those expressed in the testis (Figure 4.2; Supplementary File 1). This

result is consistent with previous morphological studies conducted within the *D. melanogaster* species complex that found that sexual traits (e.g., genital arch area, testes length and area) show consistent, statistically significant differences between species, whereas non-sexual traits (e.g., wing length and width, tibia and femur length, and malpighian tubules length and area) do not (Civetta and Singh 1998). Numerous studies have found that genes involved in sex and reproduction diverge rapidly under the effect of positive selection (Civetta and Singh 1995; Swanson and Vacquier 2002; Singh and Kulathinal 2000, 2005; Artieri et al. 2008) and, more specifically, that genes with sex-biased expression show greater evidence of positive selection than non sex-biased genes (Pröschel et al. 2006; Baines et al. 2008). Thus there appears to be evidence that the accelerated evolution observed in later stages of development is driven by unique selective pressures such as sexual selection (but see also Wade 1998; Cruickshank and Wade 2008 for examples of theory and empirical evidence suggesting relaxed selective constraint has a large effect in explaining the rapid evolution of genes with sex-limited expression).

Darwin's (1871) hypothesis that selection opportunity increases over the course of ontogeny also requires that the effects of selective pressure should only be observed at the stage in which the pressure occurs, and for which he presented secondary sexual traits as an example. While few studies have analyzed the rate of evolution of embryonic genes (Castillo-Davis and Hartl 2002; Cutter and Ward 2005; Davis et al. 2005), numerous analyses have shown that adult traits and genes involved in reproduction, particularly in male reproductive organs, often evolve at accelerated evolutionary rates when compared to most other tissues (Eberhard 1985; Haerty et al. 2007; Civetta and Singh 1995; Swanson and Vacquier 2002; Singh and Kulathinal 2000, 2005; Artieri et al. 2008). As expected, we observed that genes expressed in the pooled gonads of the adult fly are evolving more rapidly than non-gonadal adult tissue (Figure 4.2A; Supplementary Table 6). In the case of the pooled embryonic gonads, under all specificity thresholds where the differences were statistically significant, genes classified as embryonic gonad specific are evolving less rapidly than whole embryonic tissue. Thus the situation of accelerated evolution of gonad specific genes in the adult is reversed in the embryo, suggesting that the selective forces occurring in the adult reproductive stage are acting primarily on genes expressed at that stage; or at least are not affecting the embryonic stage.

Temporal pleiotropy and protein evolution

A negative correlation between breadth of gene expression and protein divergence has been observed in taxa as distant as primates and flies (Khaitovich et al. 2005; Haerty et al. 2007; Larracuente et al. 2008) suggesting the existence of a broadly applicable mechanism constraining the divergence of genes expressed in multiple tissues. The most plausible of such mechanisms is negative selection against the deleterious pleiotropic effects engendered from mutations occurring in highly connected genes (Fraser 2005;

Lemos et al. 2005; He and Zhang 2006). Our data suggest that such a model should be extended to include temporal pleitotropy to the well-supported spatial pleiotropy observed in previous studies. We observed a clear pattern of increasing evolutionary divergence (in both d_N and d_N/d_S) with increasing stage specificity of representation (Figure 4.1; Supplementary Table 4), suggesting that genes expressed ubiquitously over the course of development are subject to similar, pleiotropy-mediated evolutionary constraints as genes that are ubiquitously expressed across different tissue types (Khaitovich et al. 2005; Haerty et al. 2007; Larracuente et al. 2008). Furthermore, our observation of a significant positive correlation between the number of stages at which genes were represented and the average number of interactions in which these genes are involved strongly suggests that temporally ubiquitously expressed genes are involved in a greater number of cellular and organismal functions than their stage specific counterparts, and could thus be under more restricted evolutionary divergence due to the large effect of deleterious mutations at these loci.

4.5 Conclusions

In conclusion, we find support for both pleiotropy mediated developmental constraint, as well as Darwin's selection opportunity having a significant impact on the rates of divergence of genes over the course of ontogeny in *Drosophila*. These hypotheses are not mutually exclusive, but rather may work in tandem, each primarily influencing different stages of development in order to explain the ontogenic patterns observed among species. Therefore, given our observations, we propose a 'constraint-early/opportunity-late' model of evolutionary divergence over ontogeny (Figure 4.3), such that the reduced divergence of early-expressed embryonic genes is primarily explained by strong purifying selection minimizing the deleterious pleiotropic consequences of mutation. The accelerated divergence of late-expressed adult genes is primarily explained by unique selective pressures driving their divergence at this stage. More data and the availability of separate larval and pupal stage specific representation information will be required in order to determine the relative contributions of constraint and selection in these mid-ontogenic stages. Finally, our data imply that we ignore a large amount of information about the evolutionary dynamics of gene divergence by studying spatial gene expression at only a single stage. Any holistic approach to understanding the evolutionary dynamics of gene divergence will have to take into account temporal pleiotropy in addition to spatial pleiotropy, and as such, more temporal information about gene expression will be required in order to generate a better understanding of evolutionary divergence in which both constraint and opportunity play a role.

4.6 Methods

Gene evolutionary rate estimates

All estimates of gene evolutionary rates were obtained from the *Drosophila* 12 Genomes Consortium (2007) sequencing/annotation project according to their Phylogenetic Analysis by Maximum Likelihood (PAML) (Yang and Neilsen 2002) estimates performed on 6 species of the *D. melanogaster* group: *D. melanogaster*, *D. simulans*, *D. sechellia*, *D. yakuba*, *D. erecta*, and *D. ananassae* (Larracuente et al. 2008). d_N, d_S, and d_N/d_S (ω in PAML) as calculated under model 0 were used in this analysis. For the EST library based developmental profile (see below) the number of genes showing evidence of positive selection at each stage and for each stage/tissue category were obtained from the FDR corrected non branch specific comparisons of models 7 and 8 in the *Drosophila* 12 Genomes Consortium (2007) dataset.

EST library based developmental profile

We obtained information about the representation of all 7,180 genes in the *Drosophila* 12 Genomes Consortium (2007) dataset that were found in all stage-specific *D. melanogaster* EST libraries in NCBI's UniGene database (release version 53) (Pontius et al. 2003; http://www.ncbi.nlm.nih.gov/unigene). EST libraries separately representing the larval and pupal stages were unavailable, therefore libraries were pooled into 1 of 3 developmental stage categories based on the stage from which they were generated: embryonic, larval/pupal, and adult. Genes were then classified into developmental stages based on the stage in which they showed their highest proportion of representation among sequenced ESTs (i.e., the number of sequenced ESTs from each gene divided by the total number of ESTs sequenced in that stage's pooled libraries). Genes were re-classified into developmental stages using a series of arbitrarily chosen specificity thresholds, such that in order for a gene to be classified as specific to a stage, its highest proportion of representation had to occur at that stage and also exceed the proportion of representation at any other stage by a threshold of more than 2 fold, 4 fold, or 8 fold. Genes were also classified into a 'unique' category if they were represented only in libraries generated from a single stage, therefore producing a series of five separate sets of genes assigned to specific developmental stages (Table 4.1; Supplementary Table 1).

EST libraries from the embryonic and adult stages were separated into those derived specifically from the gonads and those derived from whole embryos (including the gonads) in the case of the embryo, and from all other tissues (not including the gonads) in the case of the adult. Genes were then classified into embryonic general, embryonic gonads, adult general, and adult gonads as indicated above, using the same specificity thresholds. In the case of the adult stage, testis and ovary derived libraries were either classified together as 'adult gonads' or separated into 'adult testis' and 'adult ovary' categories. For the purposes of this comparison, all genes classified as

larval/pupal-specific were ignored. The number of genes classified into each category and proportion of representation threshold from the EST analysis is shown in Table 4.1. In the comparison of adult and embryonic gonads and non-gonadal tissue, it is important to note that the numbers of genes classified into each category varies based on whether the adult gonads are combined or separated, especially at lower specificity thresholds, owing to the change in proportional representation introduced when the testis and ovary libraries are pooled.

FatiGO Validation of EST-based classification

We obtained NCBI 'CG' numbers for all stage classified genes for which they were available (7,027 genes) using the 'Symbol: Symbol Synonyms' tag in Flybase's (http://flybase.org) batch download feature. In the case where an FBgn was associated with multiple CG numbers, the CG number presented under the 'annotation symbol' heading of that FBgn's 'Gene Report' page was used. The few duplicate CG numbers occurring due to multiple FBgns linking to the same CG number were not removed. These duplicates most likely result from the splitting of what was originally a single gene into 2 when genome projects are reannotated. The list of CG numbers classified as specific to each stage were compared to one another using FatiGO from Babelomics 3.1 (Al-Sharour et al. 2004, 2007; http://babelomics.bioinfo.cipf.es/EntryPoint?loadForm=fatigo), searching for over-representation of GO – Biological Processes in *Drosophila melanogaster* using a two-tailed Fisher Exact Test without duplicate filtering. Only significantly over-represented terms at GO levels 3 and 4 were collected.

Developmental profile of interactions

We collected protein and gene interaction data for the 4,422 genes from the EST dataset (Supplementary Table 1) that were represented in the Biogrid database (release 2.0.36) (Stark et al. 2006; http://www.thebiogrid.org/). The total number of interactions, irrespective of the experimental methodology used to obtain them, that each gene was involved in was compiled and used in the analysis. We also compiled a dataset limited only to those interactions derived from yeast two-hybrid experiments for the purpose of ascertaining potential artifacts generated by biased stage sampling of genetic interactions (see Results) (Supplementary Table 7). Finally, we also analyzed the dataset using only 'high-confidence' yeast two-hybrid interactions as defined by Giot et al. (2003) (i.e., those interactions with a confidence score greater than 0.5).

Statistics

All statistical analyses were performed using the R statistical package (R Development Core Team 2004). Permuted Kruskal-Wallis rank sum tests and 95%

confidence intervals were computed using 10,000 permutations of the data using the 'coin' and 'boot' packages, respectively. Pairwise comparisons of the proportion of genes under positive selection were performed using chi-square tests. A Bonferroni correction for the effect of multiple tests was applied to all pairwise comparisons.

4.7 Abbreviations

CI, confidence interval; CUB, Codon Usage Bias; d_N, number of non-synonymous substitutions per non-synonymous site; d_S, number of synonymous substitutions per synonymous site; EST, Expressed Sequence Tag; MYD, million years diverged; NCBI, National Center for Biotechnology Information; NSERC, Natural Sciences and Engineering Research Council of Canada; PAML, Phylogenetic Analysis by Maximum Likelihood.

4.8 Author's Contributions

CGA, WH, and RSS conceived of the study and drafted the manuscript. CGA and WH collected data for analysis. CGA carried out the data analysis and interpretation.

4.9 Acknowledgements

We are grateful to Ben Evans, Alberto Civetta, Rob Kulathinal, and three anonymous reviewers for their insightful comments on early versions of this manuscript. This work was funded by a Natural Sciences and Engineering Research Council of Canada (NSERC) Post-Graduate Doctoral Scholarship to CGA and an NSERC grant to RSS.

4.10 Appendix: Reanalysis of Arbeitman et al. microarray dataset using the stage classification approach used on the EST dataset.

Davis et al. (2005) used the results of a cDNA microarray based study of expression of 4,028 genes over the course of *D. melanogaster* development (Arbeitman et al. 2002) and found that expression level in the late embryonic stage was negatively correlated with gene divergence between *D. melanogaster* and *D. pseudoobscura*, while it was positively correlated with gene divergence in later stages. We sought to test the generality of their results using divergence estimates obtained from orthologs within the more closely related *D. melanogaster* group (*Drosophila* 12 Genomes Consortium 2007) and using a similar approach as that described in our analysis of the EST dataset (see Methods).

Data collection

We obtained raw expression datasets from Arbeitman et al.'s (2002) study of gene expression over the course of *Drosophila* development (Gene Expression Omnibus Accession #GDS191) and concatenated all of the non tudor mutant fly array values for each of the 2,168 genes on the array for which we could obtain FlyBase (http://flybase.org) identifiers (FBgns) represented among the *Drosophila* 12 Genomes Consortium (2007) data. Each gene was classified into 1 of either 4 developmental stages (embryonic, larval, pupal, and adult) or 5 developmental stages (where adult is separated into either adult male or adult female) based on the stage at which it shows its highest level of expression (Supplementary Table 9). The entire dataset of genes was then re-classified into the same stages using arbitrarily chosen specificity thresholds such that in order for a gene to be classified as specific to a stage, its highest level of expression had to occur at that stage and exceed the next highest level of expression measured at any other stage by more than a 0.5 fold or 1.0 fold expression difference. The number of genes classified into each category and expression threshold is shown in Table 4.3. Note that the 'adult male' and 'adult female' categories do not add up to the value given in the 'adult combined' category owing to genes whose expression level is similar in both males and females and thus cannot be classified as specific to either category at given specificity thresholds.

Reanalysis of Arbeitman et al.'s microarray data

In their analysis of Arbeitman et al.'s (2002) microarray based developmental profile of expression, Davis et al. (2005) found that expression level in the late embryonic stage was negatively correlated with gene divergence between *D. melanogaster* and *D. pseudoobscura*, while it was positively correlated with gene divergence in the adult male.

We found significant differences in terms of substitution rates (d_N, d_S, and d_N/d_S) between stages under all thresholds (Kruskal-Wallis permuted rank sum test, $p < 0.01$ in all cases; Table 4.4). To examine the evolutionary dynamics of specific stages, we performed Bonferroni corrected pairwise Kruskal-Wallis permuted rank sum test comparisons of the distributions of rates of sequence divergence between all stages for each of the three specificity thresholds (i.e., no threshold, greater than 0.5 fold, and greater than 1.0 fold expression difference) (Supplementary Table 10). Genes expressed at their highest level in adults evolve more rapidly than those expressed during embryogenesis at all three specificity thresholds in terms of d_N, and d_N/d_S ($p < 2.2 \times 10^{-16}$; Table 4.5; Supplementary Table 10). Adult genes also evolve more rapidly than those of the pupal stage at all three specificity thresholds in terms of d_N and d_N/d_S ($p < 0.05$; Table 5; Supplementary Table 10). However, we found no significant differences in the comparisons of the distributions of d_N or d_N/d_S between other stages, except for a higher d_N in the adult as compared to the larval stage at no specificity threshold ($p = 0.0377$).

When we separated genes classified into the adult stage into those expressed at higher levels in males or females, we found that only those classified as adult male specific were evolving more rapidly than earlier stages (Supplementary Table 10). In this case, the comparison between the adult male stage and the larval stage was statistically significant in terms of the d_N and d_S at no expression threshold ($p < 2.2 \times 10^{-16}$ and $p = 0.0117$, respectively) and at a threshold of greater that a 0.5 fold expression difference ($p = 0.0234$ and 0.0364, respectively). The rate of evolution of genes classified as adult female specific was not statistically significantly different from that of earlier stages.

Comparison between analyses

Using correlation analysis Davis et al. (2005) found a negative correlation between gene expression and sequence divergence (d_N) in the late embryo, while they observed a positive correlation in the adult male. This supports the notion that genes follow the same pattern observed in morphology over development: genes expressed primarily in earlier stages are more conserved than those expressed in later stages. Our reanalysis of the data support accelerated sequence divergence in genes expressed primarily in adult males relative to earlier developmental stages (as well as relative to genes expressed primarily in adult females) (Supplementary table 10). The exception to this observation is the comparison between the adult and larval stage, where adult divergence was only significantly greater in the comparison of the d_N using no specificity threshold ($p = 0.0377$). It should be noted however, that there were fewer genes classified as being specific to the larval stage than the other developmental stages (Table 4.3), and this is likely limiting the statistical power of the analysis, especially at greater specificity thresholds. Our observation of fewer genes having their highest level of expression in the larval stage is somewhat puzzling given recent findings that codon usage bias (CUB) is highest in the larval stage, relative to other stages, in both *D. melanogaster* and *D.*

pseudoobscura (Vicario et al. 2008). We may expect that if CUB is being maintained due to selection for translational efficiency, the stage with the highest CUB would have an overabundance of transcripts relative to other stages. It is possible that the limited size of our dataset (2,186 genes in the *Drosophila* 12 Genomes Consortium [2007] dataset) or the limited number of genes probed in the original microarray experiment (4,028 or ~32% of the *D. melanogaster* predicted *D. melanogaster* transcriptome [Stark et al. 2007]) is leading to a bias in the number of genes that are expressed at their highest level in the larval and pupal stages.

In general, we also noted and absence of significant difference in the mean rates of divergence, in terms of d_N and d_N/d_S, of genes in comparisons among earlier stages (i.e., embryonic, larval, and pupal). Given that our analysis of the larger, EST based dataset revealed statistically significant differences in the rates of evolution between classified as being specific to the embryonic and larval/pupal stages, it is likely that the lack of significant differences among microarray based stage classifications reflects the small size of the dataset rather than the absence of actual differences. We did note a significantly lower d_S in the pupal stage as compared to the embryonic stage, which supports Vicario et al.'s (2008) observation that CUB is higher in the pupal stage than in the embryonic stage. Therefore the lower d_S in the embryo likely reflects reduced divergence due to selection for optimal codons (Akashi 2001).

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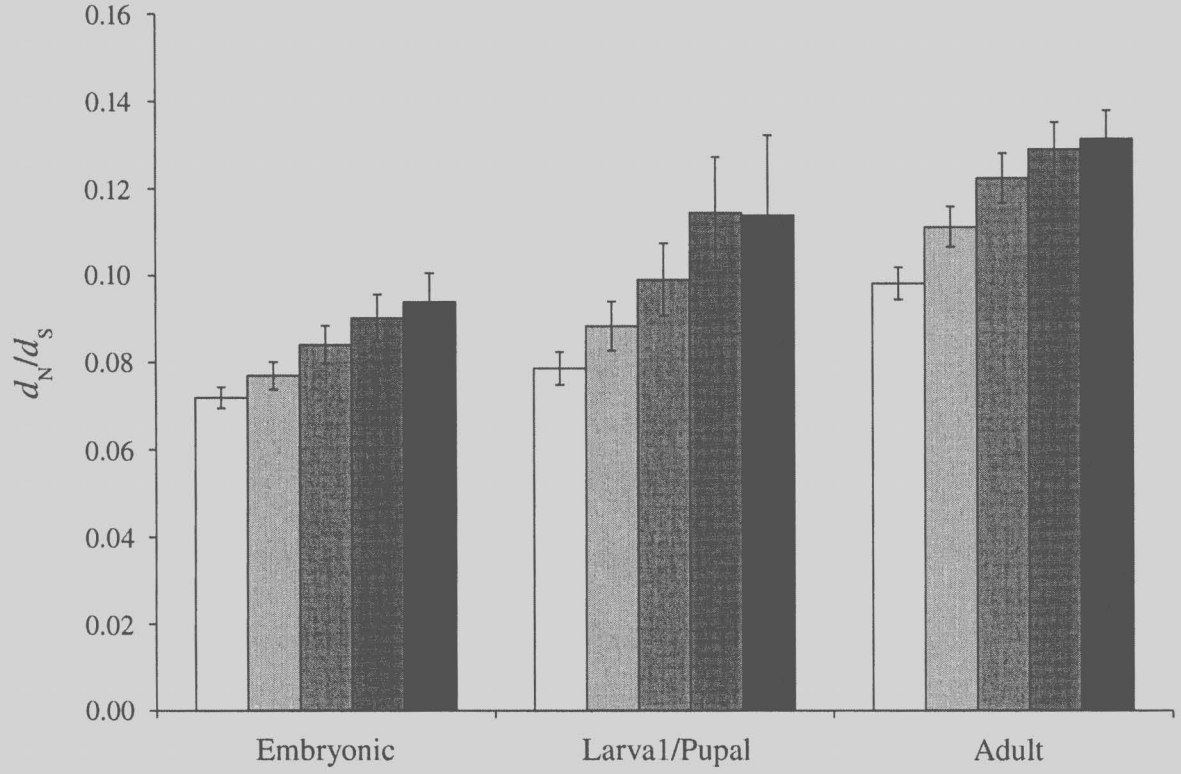

Figure 4.1: Average d_N/d_S values for genes classified into developmental stages based on EST data. Averages are shown with permuted 95% confidence intervals for each specificity threshold: (from left to right, in increasing contrast): No specificity threshold, greater than 2-fold, 4-fold, or 8-fold proportion of representation relative to other stages, and unique to a single developmental stage. Larval/Pupal represents the pooled larval and pupal stages. The differences in the distributions between stages within a specificity threshold were found to be statistically significant for most thresholds ($p < 0.01$). Furthermore the differences between thresholds within a stage were also found to be statistically significant in most pairwise comparisons ($p < 0.05$) (Supplementary Table 4).

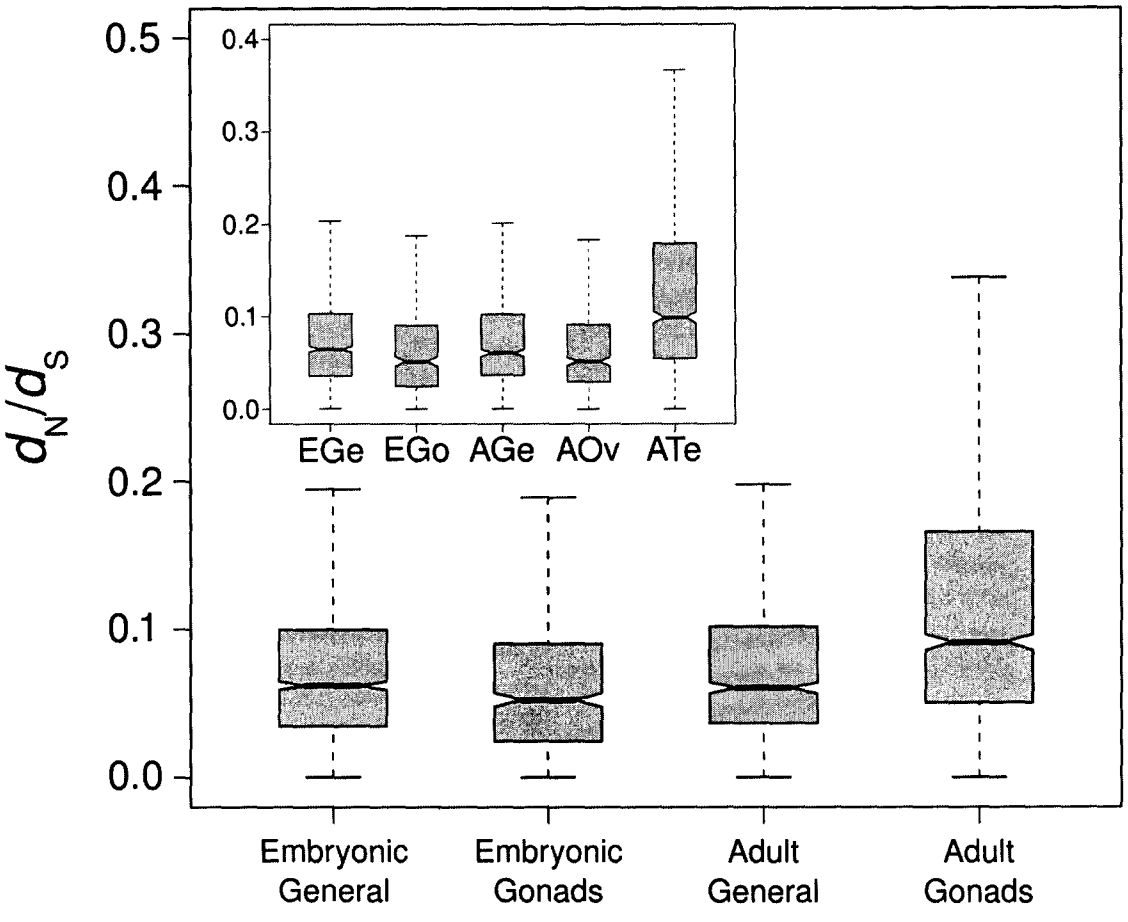

Figure 4.2: Box plot of d_N/d_S distributions for genes classified into gonadal or non-gonadal categories in the embryonic and adult stages based on EST data. Classification of genes using a greater than 2-fold proportion of representation relative to other stages is shown. Inset indicates d_N/d_S distributions when adult stage gonads are separated into ovary and testis. Abbreviations: EGe, Embryonic General; EGo, Embryonic Gonads; AGe, Adult general; AOv, Adult Ovary; ATe, Adult Testis. Genes classified into the Adult Gonads category are evolving more rapidly than all other categories ($p < 0.01$) though this is only the case for the Adult Testis category when the gonads are classified separately. On the contrary, genes classified in the Embryonic Gonads category are evolving less rapidly than all other categories ($p < 0.05$). Non-gonadal embryonic and adult tissues show no significant differences in their rates of evolution ($p > 0.05$).

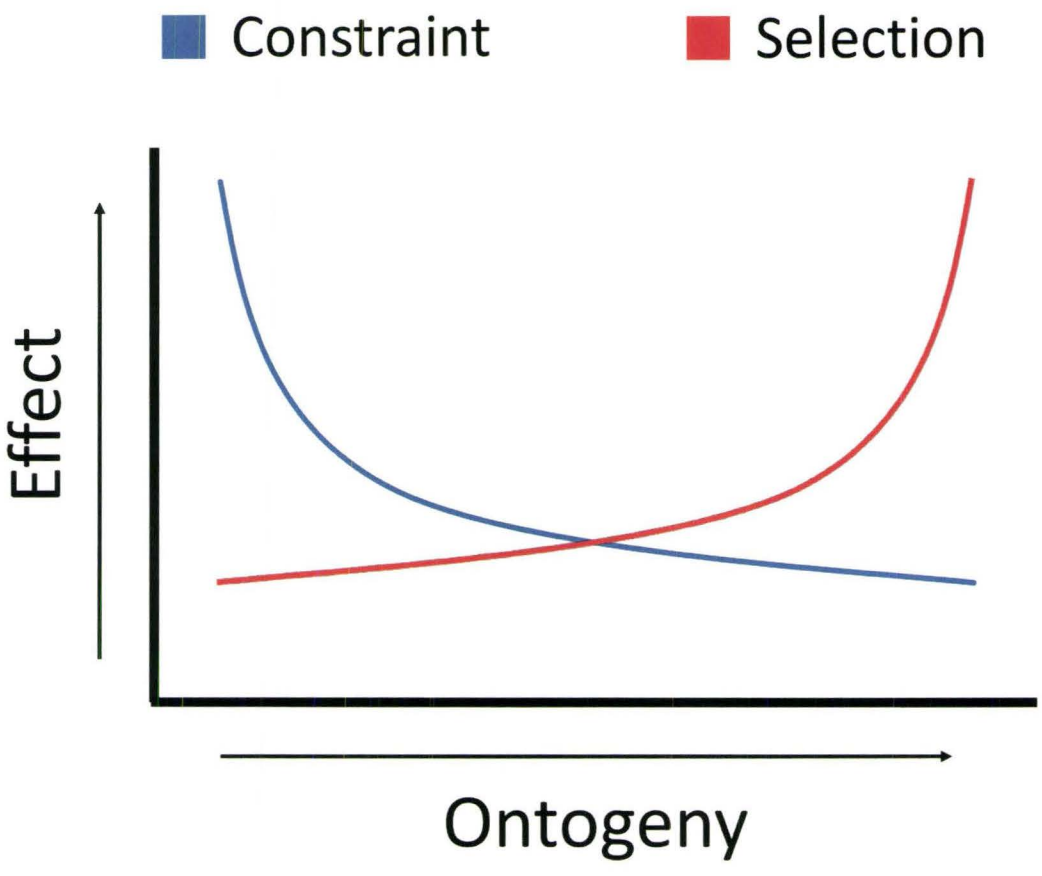

Figure 4.3: Constraint-Early/Selection-Late model of developmental divergence. Reduced divergence rates of embryonic genes relative to those of later stages are explained by purifying selection against the deleterious pleiotropic effects of mutation. Later stage genes are not simply 'less-constrained', but experience unique selective pressures, such as sexual selection, driving accelerated divergence.

Table 4.1: Number of genes classified into each category according to the proportion of representation specificity thresholds used to classify the EST data. Larval/Pupal represents the pooled larval and pupal stages. None: No threshold; > 2 fold/ > 4-fold/ > 8-fold: greater than 2-fold, 4-fold, or 8-fold proportion of representation relative to other stages; Unique: genes that are unique to a single developmental stage (see Methods).

	Specificity Threshold				
Stage	None	> 2 fold	> 4 fold	> 8 fold	Unique
Embryonic	3256	2171	1342	959	725
Larval/Pupal	1358	739	392	205	100
Adult	2566	1834	1427	1259	1191
Stage/Tissue – Gonads Combined					
Embryonic General	2234	1375	855	654	570
Embryonic Gonads	905	520	293	117	39
Adult General	1284	794	593	500	468
Adult Gonads	1688	1154	817	623	402
Stage/Tissue – Gonads Separated					
Embryonic General	1904	1144	756	636	570
Embryonic Gonads	775	415	221	103	39
Adult General	1138	754	576	496	468
Adult Ovary	1057	503	202	92	27
Adult Testis	1388	1011	779	617	367

Table 4.2: Average number of interactions (95% CI Limits) per stage and per gonadal or non-gonadal categories in the embryonic and adult stages. Classifications using no threshold and greater than 2 fold proportional representation threshold are shown. (n) indicates the number of genes in each category. Larval/Pupal represents the pooled larval and pupal stages. The average number of interactions per stage was found to be statistically significantly higher in the Embryonic stage as compare to the other two stages ($p < 0.5$). When the Adult stage is separately classified into General, Ovaries, and Testis categories, only the General and Testis categories show a statistically significantly fewer average number of interactions than the Embryonic categories, when significant (Supplementary Table 7).

	No threshold			Greater than 2 fold		
Stage	n	Mean	95% CI	n	Mean	95% CI
Embryonic	2159	8.495	(7.884 – 9.107)	1415	8.91	(8.097 – 9.729)
Larval/Pupal	858	7.401	(6.566 – 8.240)	439	6.76	(5.611 – 7.915)
Adult	1405	6.955	(6.291 – 7.619)	926	6.46	(5.713 – 7.201)
Stage/Tissue (adult gonads combined)						
Embryonic General	1504	8.762	(8.038 – 9.483)	890	9.270	(8.219 – 10.339)
Embryonic Gonad	609	8.612	(7.176 – 10.039)	331	7.254	(6.039 – 8.476)
Adult General	682	6.701	(5.947 – 7.453)	381	6.071	(5.134 – 7.018)
Adult Gonad	973	6.864	(6.128 – 7.603)	635	6.543	(5.639 – 7.453)
Stage/Tissue (adult gonads separated)						
Embryonic General	1280	8.96	(8.136 – 9.784)	721	9.51	(8.264 – 10.749)
Embryonic Gonad	520	8.73	(7.086 – 10.346)	263	7.57	(6.060 – 9.077)
Adult General	583	6.49	(5.698 – 7.278)	360	6.09	(5.104 – 7.073)
Adult Ovary	713	7.55	(6.683 – 8.419)	334	7.51	(6.298 – 8.722)

Table 4.3: Number of genes classified into each stage and sex at the three expression specificity thresholds used to classify Arbeitman et al.'s (2002) microarray data.

Stage	No Threshold	> 0.5 fold	> 1.0 fold
Embryonic	1262	848	433
Larval	118	38	8
Pupal	331	144	63
Adult Combined	444	258	163
Adult Male	339	199	118
Adult Female	104	21	12

Table 4.4: Permuted Kruskal-Wallis rank sum test results testing for significant differences between stages for d_N, d_S, and d_N/d_S based on Arbeitman et al.'s (2002) data.

Specificity Threshold	Substitution Rate	*p* value	χ^2 value
None	d_N	< 2.2e-16	31.6924
	d_S	< 2.2e-16	43.4711
	d_N/d_S	0.0001	22.8441
0.5 fold	d_N	< 2.2e-16	60.7738
	d_S	< 2.2e-16	61.3126
	d_N/d_S	< 2.2e-16	32.4076
1.0 fold	d_N	< 2.2e-16	63.7833
	d_S	< 2.2e-16	51.8955
	d_N/d_S	< 2.2e-16	39.9070

Table 4.5: Average substitution rates (95% CI limits) per stage at no, greater than 0.5 fold, and greater than 1.0 fold expression difference thresholds based on Arbeitman et al.'s (2002) expression data. The adult stage is presented when classified as a pool of both sexes as well as when both sexes are classified separately.

Stage		No threshold		Greater than 0.5 fold		Greater than 1.0 fold	
		mean	95% CI	mean	95% CI	mean	95% CI
Embryonic	d_N	0.116	(0.111 – 0.122)	0.121	(0.114 – 0.129)	0.124	(0.112 – 0.133)
	d_S	1.800	(1.769 – 1.832)	1.815	(1.777 – 1.854)	1.835	(1.780 – 1.886)
	d_N/d_S	0.067	(0.064 – 0.071)	0.070	(0.065 – 0.074)	0.070	(0.063 – 0.076)
Larval	d_N	0.1169	(0.098 – 0.136)	0.129	(0.088 – 0.169)	0.225	(0.104 – 0.347)
	d_S	1.7220	(1.627 – 1.826)	1.722	(1.545 – 1.900)	1.954	(1.515 – 2.389)
	d_N/d_S	0.0718	(0.058 – 0.086)	0.074	(0.053 – 0.095)	0.107	(0.067 – 0.146)
Pupal	d_N	0.1152	(0.104 – 0.127)	0.113	(0.094 – 0.131)	0.127	(0.097 – 0.162)
	d_S	1.6310	(1.573 – 1.689)	1.609	(1.523 – 1.696)	1.538	(1.412 – 1.665)
	d_N/d_S	0.0728	(0.065 – 0.081)	0.072	(0.060 – 0.085)	0.085	(0.060 – 0.109)
Adult (combined)	d_N	0.1626	(0.149 – 0.177)	0.193	(0.174 – 0.214)	0.214	(0.188 – 0.241)
	d_S	2.0010	(1.757 – 2.248)	2.209	(1.783 – 2.631)	2.389	(1.708 – 3.056)
	d_N/d_S	0.0865	(0.079 – 0.094)	0.098	(0.087 – 0.109)	0.107	(0.093 – 0.121)
Adult female	d_N	0.1074	(0.089 – 0.126)	0.121	(0.063 – 0.179)	0.098	(0.055 – 0.142)
	d_S	1.6840	(1.586 – 1.783)	1.812	(1.589 – 2.038)	1.897	(1.577 – 2.218)
	d_N/d_S	0.0646	(0.053 – 0.076)	0.067	(0.034 – 0.100)	0.054	(0.030 – 0.077)
Adult male	d_N	0.1798	(0.163 – 0.197)	0.203	(0.180 – 0.226)	0.227	(0.196 – 0.259)
	d_S	2.1000	(1.782 – 2.420)	2.322	(1.782 – 2.861)	2.543	(1.638 – 3.451)
	d_N/d_S	0.0934	(0.084 – 0.103)	0.102	(0.090 – 0.114)	0.113	(0.096 – 0.130)

CHAPTER 5

Drosophila hybrids show conserved gene regulation during metamorphosis amid ontogenic stage-specific autonomy of misexpression

This chapter is currently being formatted for submission for publication. In this study, we extracted whole-body male mRNA from three species of the *Drosophila melanogaster* group (*D. melanogaster*, *D. sechellia*, *D. simulans*) as well as *D. simulans* (♀) × *D. sechellia* (♂) F1 hybrids at 4 different developmental stages (3rd instar larval, early pupal, late pupal, and newly-emerged adult) and profiled gene expression levels using *D. melanogaster* cDNA microarrays representing approximately 10,000 unique genes. We wanted to assess whether a) earlier-stage gene expression profiles were more conserved between species as was observed for gene coding sequence (see Chapter 4), and b) if fewer genes were misexpressed in the hybrids during earlier stages (see Chapter 3), as would be expected if there was greater conservation of regulatory factors during earlier stages of ontogeny. I conceived of the study with the assistance of my supervisor and W. Haerty. I also performed the majority of the tissue collection, though I was greatly aided during the summer of 2007 by Mariša Melas, an undergraduate research assistant. I performed all data analysis and drafted the chapter with the editorial assistance of my supervisor and W. Haerty. Please note that supplementary materials are provided on the compact-disc distributed with the thesis.

5.1 Abstract

The body of speculation about the relative importance of changes in gene regulation in determining broad phylogenetic patterns continues to accrue, despite a lack of broad-scale, comparative studies examining how patterns of gene expression vary over development. Several studies have, however, examined expression patterns in adult interspecific hybrids, uncovering patterns in divergence of the mechanisms controlling gene regulation. This has led to the suggestion that the widespread misregulation of gene expression observed in adults results from the downstream cascading effects of a smaller number of genes improperly regulated in early development. We sought to test this hypothesis by transcriptional profiling of males of three species of the *Drosophila melanogaster* group (*D. melanogaster*, *D. sechellia*, and *D. simulans*) as well as the *D. simulans* (♀) × *D. sechellia* (♂) male F1 hybrids at 4 different developmental stages (3rd instar larval, early pupal, late pupal, and newly-emerged adult). Contrary to the cascading model of hybrid misexpression, we find that there is considerable stage-specific autonomy of regulatory breakdown, with the 3rd instar larval and adult stages showing significantly more hybrid misexpression as compared to the pupal stages. Furthermore, genes expressed at earlier stages of development tend to be more conserved in terms of expression level than those expressed at later stages, supporting previous morphological and nucleotide sequence based observations. Our results suggest that complex integration of regulatory circuits during morphogenesis may lead to it being more refractory in terms of divergence of gene regulatory factors, supporting a 'developmental hourglass' model of divergence of gene expression in *Drosophila* wherein earlier and later stages are more free to diverge in comparison to a highly conserved pupal stage.

5.2 Introduction

Studies in the field of evolutionary developmental biology (evo-devo) have highlighted an important role for divergence in patterns of gene regulation in shaping species-specific developmental outcomes (reviewed in Prud'homme et al. 2007, Carroll 2008). The majority of interspecific studies in this field have focused on one or few loci, employing molecular techniques developed through the study of developmental genetics, with the intent of mapping the precise changes in *cis* regulatory elements that are directly responsible for altered phenotypes (reviewed in Carroll 2005, Wray 2007). Conversely, large-scale inter-species comparative gene-regulation/expression studies in the context of development are lacking, despite a growing body of speculation on the importance of divergence in gene regulatory networks and hierarchies in determining broad evolutionary patterns (Davidson et al. 2003; Davidson and Erwin 2006; Coyne 2006; Coyne and Hoekstra 2007). Such comparative studies are crucial to a more complete synthesis of evolution and development, as theoretical models of evolutionary processes ultimately

derive from the attempt to explain general patterns, rather than lists of case studies (Gould 2002).

A classic example of such a broad pattern related to the evolution of development involves Karl Ernst Von Baer's (1828) famous 'third law of development', which can be summarized in the observation that species are more similar to one another in earlier developmental stages as compared to later stages (Gould 1977; Raff 1996). Modern hypotheses seeking to explain the greater similarity of morphology between species at early developmental stages have generally invoked the notion that mutations in early-expressed, developmentally important genes will be more likely to have deleterious cascading effects throughout ontogeny as compared to genes expressed in later stages, thus resulting in stronger purifying selection limiting the divergence of early-expressed genes – also known as 'developmental constraint' (Stebbins 1974; Riedl 1978). More recently, it has been suggested that while developmental constraint may explain conservation of sequence and structure in early development, greater opportunity for selection in later stages of development, engendered by such features as greater organismal mobility, complexity of behavior, sexual reproduction, etc., may also contribute to the greater level of divergence seen among species' adults (Artieri et al. 2009) – a theory originally proposed by Darwin (1872; 1882). It is also bears noting that while Von Baer's third law holds generally, it is now well established that interspecific divergence does not increase monotonically over the entire course of development; rather, the very earliest stages of ontogeny in vertebrates, for example, can vary substantially between species (see Raff 1994, 1996 for review). This has led to the proposal of a 'developmental hourglass' model of divergence over ontogeny, wherein certain stages of development are more highly conserved as a result of a greater integration of complex regulatory interactions as compared to those occurring either earlier or later (Raff 1996). This hypothesis rests on the prediction that qualitative differences exist in the organization of developmental genetic pathways during different stages of ontogeny, with the earliest processes involving establishment of simple, global patterns, the intermediate, conserved, stages involving high levels of inter-connectivity as organ primordia are established, and finally later stages becoming more modular as organs begin their own, isolated developmental trajectories (Raff 1994).

Evidence for greater conservations of genes expressed during earlier stages of development, as well as greater opportunity for selection to act on genes expressed during later stages, has been provided by a variety of recent studies that have analyzed genomic data sets. For instance, genes with higher levels of expression in earlier developmental stages as compared to later stages have been found to be more conserved in their coding sequence as compared to genes with opposite patterns of expression (i.e., higher in adults) in both *Drosophila* and *Caenorhabditis* (Davis et al. 2005; Cutter and Ward 2005). Expanding upon these previous studies, Artieri et al. (2009) found a linear increase in the rates of coding sequence divergence in the *Drosophila melanogaster* subgroup over the span of ontogeny among genes classified into specific developmental stages based on

their representation in stage-specific EST libraries. Furthermore they noted that genes expressed during embryogenesis were, on average, involved in a significantly higher mean number of interactions than genes expressed at later stages, consonant with the notion that these genes evolve less rapidly due to purifying selection acting against deleterious pleiotropy created by mutations in these highly-connected genes. Supporting the selection opportunity hypothesis, all three of these studies noted that the accelerated evolution observed in later stages was largely a consequence of rapid divergence of male-biased (expressed at higher levels in males as compared to females) or spermatogenesis-specific genes, both of which show evidence of frequent, recurrent positive selection, presumably as a consequence of being important targets of sexual selection (see Singh and Kulathinal 2000; Swanson and Vacquier 2002; Ellegren and Parsch 2007 for review). Studies have also found support for the developmental hourglass model as well. Galis and Metz (2001) conducted a wide-ranging literature review and found abundant evidence that the vertebrate phylotypic stage is the most sensitive to external perturbation, as well as the most likely stage where such perturbation will cause lethality. Such consistent documentation of increased lethality during this conserved stage further agrees with the hypothesis that its conservation is the result of purifying selection limiting the divergence of highly connected, developmentally integrated genes.

Studies of interspecific hybrids have noted that such hybrids often display aberrant aspects of development, such as atrophied or absent germlines, or heterosis of particular tissues/organs (Parker et al. 1985; Voss and Schaffer 1996; Coyne and Orr 2004). Consequently, many researchers have employed interspecific hybrids in order to explore questions pertaining to the evolution of development, particularly with regards to how divergence of mechanisms regulating gene expression level may manifest themselves as hybrid dysfunctions in the context of proper parental ontogeny (see Ortíz-Barrientos et al. 2007 for review). In the past decade, the focus of these studies has shifted from the analysis of single genes towards examining transcriptome-level patterns (see Ranz and Machado 2006; Ortíz-Barrientos et al. 2007 for review), and, despite various methodologies and experimental platforms, these studies have agreed upon at least one significant observation: quantitative divergence between hybrids and same sexed members of their parental species, manifested as improper expression of genes within hybrids relative to both parental species (termed misexpression) is widespread, and the degree to which genes are misrexpressed in hybrids is positively correlated with their rates of sequence divergence between parental species (Artieri et al. 2007). The majority of genes misexpressed in interspecific hybrids are underexpressed relative to their parents, which is thought to result from a loss-of-function phenotype in hybrids created by incompatible divergence of gene regulatory elements (Ranz et al. 2004). However, a potential caveat to studies of gene expression in interspecific hybrids is that the majority of them have been performed examining only a single developmental stage: typically the adult. Given that development is regulated by complex developmental cascades (see above), it has been suggested that the widespread misexpression observed in adults may

not reflect equally widespread, incompatible divergence of regulatory elements during a single developmental stage, but rather may result from incompatibilities occurring among a much smaller number of genes upstream in these hierarchies having complex cascading effects that manifest themselves later in adults.

With the intent of exploring how gene regulation diverges between species over development, we have conducted whole-transcriptome cDNA microarray-based expression profiling of males in 3 species of the *Drosophila melanogaster* subgroup (*D. melanogaster*, *D. sechellia*, and *D. simulans*) at four synchronized developmental time-points (3rd instar larval, early pupal, late pupal, and newly-emerged adult). In addition, we also performed the same analysis on the male interspecific F1 hybrids of the *D. simulans* (♀) × *D. sechellia* (♂) cross with the intent of addressing the following outstanding question with respect to the evolution of gene expression in the context of development: Are patterns of expression more conserved between species in earlier developmental stages as Von Baer's observations would predict? This could also manifest itself as a reduction in the proportion of genes that are misexpressed in interspecific hybrids due to greater conservation of their underlying regulatory elements. Answering this question will allow discrimination between the hypothesis that hybrid misregulation results from the cascading effect of the improper expression of early-expressed developmental effectors versus stage-specific incompatible divergence of regulatory factors.

5.3 Materials and Methods

Time-synchronized collection of *Drosophila*

Collection of *D. melanogaster* (14021-0231.00), *D. sechellia* (Cousin Island, Jean R. David, Centre National de la Recherche Scientifique, Gif sur Yvette, France), and *D. simulans* (14021-0251.2) individuals proceeded as follows: Approximately 30 individuals of each species (males and females) were placed overnight at 25°C on a 10 cm plastic petri dish, which was approximately half-full of standard cornmeal – molasses – agar medium upon which a small amount of yeast mixed with lukewarm tap water was painted with a small paintbrush. Each dish was covered by a 100 ml tri-cornered beaker into which small holes had been punctured to assure adequate ventilation while preventing escape of the flies. In the morning, the flies were transferred to a fresh yeast-painted petri dish, while all larvae on the dish from the previous day, visible by inspection under a dissecting microscope, were removed using a syringe. The dish was then allowed to incubate again at 25°C for 2 hours at which point all newly emerged larvae were transferred onto fresh cornmeal-molasses - agar dishes and placed at 25°C for 96 hours. No more than 30 individuals were placed on a single plate in order to prevent density dependent growth effects. At 96 hours, during the 3rd instar larval stage, individuals were sexed under a dissecting microscope based on the morphology of the developing gonads

(Ashburner 1989), and males were anesthetized on ice in RNALater (Ambion) and placed at -80°C until mRNA extraction (see below).

After 200 individuals of each species had been collected, a similar protocol was followed except that in this case after 30 individuals of each species were placed overnight at 25°C on a 10 cm plastic petri dish, all larvae found in the morning were transferred to fresh cornmeal – molasses –agar petri dishes, again with no more than 30 individuals per dish, and allowed to develop at 25°C for approximately 120 hours. Dishes were then examined for the presence of pupae, which were discarded. After 2 hours, any larvae that had begun to undergo pupation (as defined by the presence of a brown puparium and complete lack of movement) were sexed, transferred to fresh petri dishes and allowed to develop for 2 (early pupal stage) or 72 (late pupal stage, post red eye) more hours before collection of males on ice in RNALater and immediate transfer to -80°C until mRNA extraction. Virgin adults were collected by tapping out pupae-containing jars in the morning, and collecting any flies that had emerged in the following 1.5 hours. Individuals were sexed based on genital morphology, anesthetized on ice in RNALater and transferred immediately to -80°C until mRNA extraction.

Collection of *D. simulans* × *D. sechellia* hybrids

Approximately 10 4-day old *D. simulans* (14021-0251.2) virgin females were placed with 15 newly emerged *D. sechellia* (Cousin Island, Jean R. David Centre National de la Recherche Scientifique, Gif sur Yvette, France) males on petri dishes following the protocol described above. However, unlike in the case of the pure species, newly emerged F1 hybrid flies were never discarded, but rather placed into a separate glass vial with cornmeal-molasses-agar medium and kept for approximately 20 days. Given that the F1 hybrid males from this cross are sterile (Bock 1984), any larvae observed in the vial after 20 days indicated that at least one the parental females was not a virgin, and thus all individuals collected from that cross were discarded.

mRNA extraction and microarray hybridization

25 males from each stage and species/hybrid were collected and mRNA was extracted using the RNeasy Mini kit (Qiagen). Given that it was impractical to collect sufficient mRNA from hybrids for direct use in microarray hybridizations, all mRNA samples were then amplified twice using the MessageAmp II aRNA kit (Ambion). A much larger amount of *D. melanogaster* mRNA was extracted from each stage in order to use as an equal concentration mixed-stage (unamplified) reference on the cDNA microarrays. All samples as well as the reference were sent to the Canadian Drosophila Microarray Centre (CDMC, http://www.flyarrays.com) for hybridization on *Drosophila melanogaster* 12Kv2 cDNA microarrays spotted with ~12,000 elements representing approximately 10,000 unique genes. In the case of *D. melanogaster*, *D. sechellia*, and *D.*

simulans, the amplified mRNA from a single pool of 25 male flies was hybridized on three technical replicate microarrays according to the protocols below. However, in the case of the *D. simulans* × *D. sechellia* hybrids, mRNA from three separately extracted and amplified pools of 25 males were each used for hybridization to a single microarray, such that the replicates were also biological as well as technical, in order to determine whether there was a significant effect of between-extraction/amplification variability on our estimates of expression differences (see below).

In the case of the amplified samples, the following reverse transcription protocol was used: RNase-free water was added to 5 μg of total RNA from each sample to bring the final volume to 14.5 μl. 4 μl of random primer was added followed by incubation at 70°C for 10 min, then 42°C for 5 min. 19.5 μl of modified indirect reverse transcriptase master mix was added to each tube, along with 2.0 μl of Superscript II RT and the reaction was incubated at 42°C for 3 hours. For the unamplified mixed-stage *D. melanogaster* reference sample, the following reverse transcription protocol was used: RNase-free water was added to 60 μg of total RNA from each sample, to bring the final volume to 19 μl. 21 μl of Indirect RT master mix was added into each tube and the reaction was incubated at 65°C for 5 min, then 42°C for 5 min. 2 μl of Superscript II RT was added to the sample, followed by incubation at 42°C for 3 hours.

The cDNA product was cleaned and precipitated by adding 8 μl of 1N NaOH to each reaction with mixing by pipetting followed by a quick spin and immediate incubation at 65°C for 10 min. 8 μl of 1N HCl was then added, followed by 4 μL of 1M Tris (pH 7.5), mixing by pipetting after each addition. 38 μl of water was added to bring the total volume to 100 μl, and the amino allyl-cDNA was purified using either the Qiagen PCR clean up or Invitrogen Purelink purification kit (using 80% EtOH for the wash buffer and eluting with 2 × 50 μl of water). After purification, 10 μl of 3M NaOAc, 1 μl of glycogen (20 μg/μl) and 120 μl of ice-cold isopropanol were added, and the cDNA was allowed to precipitate at –20°C for at least 75 min or overnight. The precipitated cDNA was then spun at >12,000 g for 30 min and the pellet was washed with 200 μl of 75% EtOH, followed by another spin at >12,000 g for 5 min. All EtOH was carefully pipetted from the tube and the probe pellet was allowed to dry for ≤ 1 min before resuspension in 5 μl of water.

Samples were then dye conjugated by addition of 3 μl of 0.3 M $NaHCO_3$ to the resuspended amino allyl-cDNA, followed by 2 μl of reactive dye (Alexa647 for samples, and Alexa555 for the reference) and subsequent incubation at room temperature in the dark for 1 hour. 90 μl of ddH2O was added to each sample, followed by purification using either the Qiagen PCR clean up or Invitrogen Purelink purification kit, washing with 80% EtOH 3 × and eluting with 3 × 50 μl of water. 15 μl of 3M NaOAc, 1.5 μl of glycogen (20 μg/μl) and 170 μl of ice-cold isopropanol were added to the labeled probe,

and the DNA was allowed to precipitate at –20°C for at least 30 min. The precipitated probe was then spun at >12,000 g for 30 min and the pellet was washed with 200 µl of 75% EtOH, and spun at >12,000 g for 5 min. All EtOH was carefully pipetted from the tube and the probe pellet was allowed to dry for ≤ 1 min before resuspension in 5 µl of water.

Microarrays were competitively hybridized using the following protocol: 80 µL of hybridization buffer (75 µl of DIG Easy Hyb [Roche], 4 µl of 10 mg/ml yeast tRNA [Invitrogen], and 4 µl of 10 mg/ml salmon sperm DNA [Sigma]) was added to each resuspended probe, followed by incubation at 65°C for 10 min. Both sample and reference probes were placed on the array, which was then placed in a sealed chamber in a 37°C water bath for 16-18 hours. The array was then washed for 3 × 15 min in pre-warmed 1 × SSC, 0.1% SDS. The array was then washed with room temperature 1 × SSC for ≤1min, followed by room temperature 0.1 × SSC for ≤ 15 sec. Arrays were scanned using a ScanArray 4000 XL (GSI Lumonics/Packard Biochips); images were preprocessed and quantified using QuantArray v3.0 (PerkinElmer).

Analysis of microarray data

Data from the scanned microarrays were uploaded into GeneTraffic™ DUO version 3.0 (Iobion Informatics) and replicate spots were filtered such that any element showing a greater than 200% coefficient of variation or a 2-fold greater difference among the highest and lowest measured replicate spot (including all elements within an array or between replicate arrays) was flagged. All elements flagged according to these criteria as well as those flagged by the internal quality control standards of the software were then manually inspected for the presence of unacceptable spots (e.g., incorrectly printed, contained visible surface scratches or matter interfering with the scanning, etc.). Such spots were removed, and if less than 6 usable replicates (i.e., 2 replicate spots per array) remained, the entire element was discarded. Furthermore, an element was discarded if a subset of the within-array replicate spots showed consistently different hybridization intensities; these spots likely represent clones that were incorrectly annotated as belonging to the same element in the CDMC's *Drosophila* 12kv2 microarray annotation file (http://142.150.8.217/GT_annot.zip). The filtered raw data was then downloaded from GeneTraffic™ DUO version 3.0 and subjected to a second round of quality control, where spots were removed if they did not show an expression intensity of at least 100, as well as a two-fold expression intensity above either the local or global average background. All genes that did not have usable data from both replicate spots on all three microarrays in all four stages within a species were then removed from further analysis. The CDMC's *Drosophila* 12kv2 microarray annotation file was then manually inspected in order to identify all spots for which a single Flybase gene number (FBgn) (FB2008_10 Dmel Release 5.13; http://flybase.org/) could unambiguously be identified (the manually

updated array spot number/clone - FBgn associations are listed in Supplementary Table 2). All control spots as well ambiguous clone spots were removed from further analysis, leaving only genes that were detectibly expressed in all 4 stages in at least one of the 3 species or the hybrid.

Normalization and microarray data analysis

The data remaining after quality control were normalized using the 'rlowess' procedure (spatial-intensity joint loess) as implemented in the 'maanova' package in the R statistical software (Wu et al. 2003; http://research.jax.org/faculty/churchill/software/Rmaanova/index.html), using default values and a 'TwoColor' array type. The output for each spot on each array from maanova was then transformed into its $\log_2$(sample/reference) ratio, which was then analyzed by the software Significance Analysis of Microarrays as implemented in the 'samr' package in R (Tusher et al. 2001; Storey and Tibshirani 2003; http://www-stat.stanford.edu/~tibs/SAM/). Differential expression of genes between samples was estimated using two methods: for within-species, between-stage comparisons (i.e., do genes vary significantly in expression over the course of development within a species?), samr was run on all genes from the dataset of 4,286 that were detectibly expressed within a species/hybrid using the 'Multiclass' response type, 2000 permutations of the data, and a False-Discovery Rate (FDR) of 5%. Furthermore, pairwise Bonferroni-corrected t-tests were performed on the distribution of replicate values for each developmental transition (3^{rd} instar larval to early pupal, early pupal to late pupal, and late pupal to adult) for all genes that were identified as varying significantly among the 4 developmental stages. Genes that did not vary significantly (as identified by the t-test) by at least a 1.5 fold expression difference in at least one of the three developmental transitions were rejected as not varying significantly over development. In the case of between pure species or between hybrids and their parental species, within-stage comparisons (i.e., are genes significantly differentially expressed between species/hybrids within a given stage?), samr was run on all genes from the dataset that were detectibly expressed in both species of a given pairwise comparison using the 'Two class unpaired' response type, a minimum-fold expression difference threshold of 1.5, 2000 permutations of the data, and an FDR of 5%. After samr analysis, all remaining genes in the analysis were inspected for duplicates (some genes are spotted in duplicate on the microarray). If a given gene showed the same pattern of expression in all of its duplicates, only the first spot in numerical order according to the annotation file, or the first spot with statistically significant differences, was retained for further analysis. Any duplicates that did not agree in expression pattern (e.g., higher in one species in one duplicate, but higher in the other species in another duplicate) were excluded from further analysis. A summary of all raw data with results of the analysis for the 4,286 genes retained in the final analysis is found in Supplementary Table 1.

Variance analysis

The variance (σ^2) was estimated for the distribution of replicates spots (within or between arrays) within each stage and for each species/hybrid, and the distribution of variances were compared to one another using pairwise, permuted Kruskal-Wallis tests (Supplementary Figure 5.1). In no case was the mean σ^2 of the *D. simulans* (♀) × *D. sechellia* (♂) F1 hybrids, which were generated using biological replicate arrays, significantly higher than all pure species (it was always significantly lower than *D. melanogaster*) confirming that the majority of biological variability in expression levels was captured in our pools of 25 males. σ^2 estimates in *D. sechellia* were significantly lower than all other species/hybrids in the case of the 3rd instar larval, early pupal, and late pupal stages (see results below). We therefore simulated *D. sechellia* expression data such that *D. sechellia* means would be maintained, but variances would be scaled to *D. simulans* levels using a custom PERL script. The mean $\log_2$(sample/reference) expression value in *D. sechellia* ($\bar{x}_{D.sec}$), as well the *D. simulans* σ^2 ($\sigma^2_{D.sim}$) for each gene among the 2,006 genes detectibly expressed in all stages in the three species and the hybrids was obtained. Three random numbers, y_1, y_2, and y_3, were then chosen such that they summed to $(n-1)/2 \times \sigma^2_{D.sim}$ (where n = 6 replicate spots). The new distribution of *D. sechellia* values was generated by creating three pairs of values, one for each of the random numbers, each equal to $\bar{x}_{D.sec} + \sqrt{y_x}$ and $\bar{x}_{D.sec} - \sqrt{y_x}$. The 6 simulated replicate array values were then used in order to reanalyze the *D. sechellia* data (see below).

Validation by quantitative reverse-transcriptase PCR, coding sequence divergence

We selected 5 genes (FBgn0004413, FBgn0032350, FBgn0032515, FBgn0033608, FBgn0034443) upon which to perform qRT-PCR at random from the dataset of expressed genes that matched the following criteria: The minimum number of significant pairwise differences (i.e., > 1.5 fold difference as assessed by SAM) among all possible between-species, within-stage pairwise comparisons had to be ≥ 10, in order to have enough comparisons to draw meaningful correlations between the microarray and qRT-PCR data (Supplementary Table 3). Given that sample mRNA was amplified, thus biasing sequences towards short 3' reads, PCR amplification primers were designed using Primer3Plus (Untergasser et al. 2007; http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) to span a ~100 bp region found on the 3' most constitutively expressed exon. The *D. melanogaster*, *D. simulans*, and *D. sechellia* sequences were first aligned in order to choose primer sites where the sequences were identical among all 3 species, and for which none of the species possessed indels in the amplified sequence (primer sequences are provided in Supplementary Table 3). Primers were first tested via gradient PCR on genomic DNA from all three species in order to make sure that they amplified correctly and determine optimal annealing temperatures. The original mRNA amplifications that were sent to the CDMC (see above) were obtained, and diluted to a concentration of 50 μg/μl, of which 5 μl was used to generate

cDNA via oligo dT priming using the AffinityScript Multiple Temperature cDNA Synthesis Kit (Stratagene). RT-PCR was then conducted on 0.8 μl of cDNA product for 2 replicates of each of the 16 samples ([3 species + hybrid] × 4 stages) using 10 μl reaction volumes with the Brilliant II SYBR Green qPCR Master Mix (Stratagene) (using a 1:500 dilution of ROX reference dye to correct for pipetting variation). Samples were loaded into 96 well ABgene PCR Plates (Thermo Scientific) and run on a Mx3000P Real-Time PCR System (Stratagene) under the following cycling protocol: 95°C for 10 min, followed by 40 cycles of 95°C for 30 sec, an annealing temperature of 63°C for 1 min, and 72°C for 30 sec. All runs were conducted with SYBR Green disassociation curves (95°C for 1 min followed by a gradual cool to 55°C and then a gradual return to 95°C) in order to confirm that primers were amplifying a single unambiguous product. Between sample fold-changes were calculated by taking the difference of the average threshold cycles (Ct) between both replicates for each sample, where fold change = $2^{\Delta Ct}$.

We further validated the results of our expression analysis by testing for a potential correlation between pairwise expression and sequence divergence among the 3 pure species. In this case we obtained the FASTA coding sequences for *D. melanogaster*, *D. sechellia*, and *D. simulans* for all genes in our dataset represented in the *Drosophila* 12 Genomes Consortium (2007) *D. melanogaster* group data. All three possible pairwise alignments were performed using Dialign-TX version 1.0.2 (Subramanian et al. 2008), followed by estimation of d_N, d_S, and d_N/d_S via codeML in Phylogenetic Analysis by Maximum Likelihood (PAML; Yang and Nielsen 2002) (Supplementary Table 1).

Divergence rates over ontogeny analysis

We obtained PAML Model 0 (a single rate for the entire tree) estimated rates of divergence (d_N, d_S, and d_N/d_S) for all genes in our dataset represented in the *Drosophila* 12 Genomes Consortium (2007) *D. melanogaster* group data (representing the six sampled species of the *D. melanogaster* group). Each gene was classified into 1 of the 4 sampled developmental stages (3rd instar larval, early pupal, late pupal, or adult) based on the stage at which it shows its highest level of expression. The entire dataset of genes was then re-classified into the same stages using arbitrarily chosen specificity thresholds such that in order for a gene to be classified as specific to a stage, its highest level of expression had to occur at that stage and exceed the next highest level of expression measured at any other stage by more than a 0.25 fold or 0.5 fold expression difference (Supplementary Table 1).

Statistical analysis

All statistical analyses were performed using the R statistical package version 2.7.2 (R Development Core Team 2004). Permuted Kruskal-Wallis rank sum tests were performed with 10,000 permutations of the data using the 'coin' package. Permuted 95%

confidence estimates were generated using the 'boot' package on 10,000 permutations of the data.

5.4 Results

Within-species variation in expression levels over ontogeny

We used *Drosophila melanogaster* cDNA microarrays representing approximately ~10,000 unique genes to measure expression patterns of male whole-body mRNA extracts of *D. melanogaster*, *D. simulans*, and *D. sechellia*, as well as the interspecific F1 hybrids of the *D. simulans* (♀) × *D. sechellia* (♂) cross (hereafter '*D. sim* × *D. sec* hybrids' or simply 'hybrids') at 4 different developmental stages (3rd instar larval, early pupal, late pupal, and adult; see Materials and Methods). *D. simulans* and *D. sechellia* shared a most recent common ancestor (MRCA) ~0.5 to 1.0 million years ago (MYA) and form a clade that shared an MRCA with *D. melanogaster* approximately 5.4 MYA (Kliman et al. 2000; Tamura et al. 2004). Comparing only genes that are detectibly expressed in the 3 species and the hybrids (2,006 genes) we found that 64.2% (1,287), 82.2% (1,649), 62.2% (1,247), and 57.9% (1,162) of genes varied significantly in expression level over the course of the four sampled developmental stages in *D. melanogaster*, *D. sechellia*, *D. simulans*, and the hybrids, respectively. The proportion of genes that varied significantly during development in *D. sechellia* was significantly greater than that of the other two species and the hybrids (χ^2 test, 1 degree of freedom [df], $P = 3.709214 \times 10^{-6}$, 1.179722×10^{-7}, and 1.642797×10^{-11} for the comparison with *D. melanogaster*, *D. simulans*, and the hybrids, respectively. Note that this and all subsequent pairwise comparisons are Bonferroni corrected for multiple tests); however, no other comparisons were statistically significant ($P > 0.05$). It should be noted that the proportion of genes varying among species is not nested, though 728 of the genes varying significantly in expression level over ontogeny are shared between all 3 species and the hybrids (representing between 44% [*D. sechellia*] and 63% [hybrids] of genes varying significantly in expression level during development); the relationships among what genes are shared among the three pure species or the parental species and the hybrids are shown in Venn diagram form in Supplementary Figure 5.2. We also compared the proportions of genes that varied at each of the three sequential developmental transitions (i..e, 3rd instar larval to early pupal [L to EP], early to late pupal [EP to LP], and late pupal to adult [LP to A]) among the three species and the hybrids (Figure 5.1A). Similar to the situation noted above, we found that *D. sechellia* shows significantly more genes varying in expression level in all three transitions as compared to the other 2 species or the hybrids (χ^2 test, 1 df, $P < 0.001$). No other comparison among pure species or the hybrids and their parental species was significantly different for any of the transitions, with the exception that there are significantly more genes varying in expression level during the L to EP transition in *D. simulans* as compared to the hybrid ($P < 0.001$).

Overall, these patterns may suggest that, in terms of the number of genes varying significantly over the course of development, the hybrids are more similar to *D. simulans* than *D. sechellia* (Figure 5.1A). In comparisons between transitions within species, the number of genes varying significantly in expression level during each transition did not vary significantly within *D. melanogaster*; however, more genes varied during the L to EP transition as compared to the EP to LP transition in *D. sechellia*, while more genes varied during the EP to LP as compared to the LP to A transition in *D. simulans* ($P < 0.01$ in both cases) (Figure 5.1A). In the hybrids, more genes varied significantly in expression level during the EP to LP transition as compared to the other two transitions ($P < 0.001$ in both cases). No other within-species comparisons were statistically significant.

The observed increase in the number of genes that vary significantly over the course of ontogeny in *D. sechellia* could be an artifact of this species having particularly low between replicate variance in expression level estimates, thus increasing our statistical power to detect differences in gene expression between samples. Therefore we compared the distributions of the between replicate variance (either replicate spots on the same array or between replicate arrays) estimates for the $\log_2$(sample/reference) expression values for all 3 species as well as the hybrids (Supplementary Figure 5.1). Indeed, between replicate variances in *D. sechellia* are significantly lower than the other species/hybrids at three stages: 3rd instar larval, early pupal, and late pupal; in the case of the adult stage, *D. sechellia* estimates are significantly greater than either *D. simulans* or the hybrids (Kruskal-Wallis rank sum test, $P < 2.2 \times 10^{-16}$ in all cases). In order to determine whether the low between replicate variance led to a significant over-estimate of the within species expression differences in *D. sechellia*, we generated a set of random array values for all 2,006 genes that were detectibly expressed in the three species and the hybrids such that the values for each gene had the same mean as those of *D. sechellia*, while their variance was equivalent to that estimated from *D. simulans* (see Materials and Methods). We then reanalyzed the data using the simulated array values and found that the number of genes varying significantly in expression level over the sampled developmental stages in *D. sechellia* was reduced from 1,649 (82.2%) to 1,446, or 72%. In this case, the number of genes that vary significantly over development in *D. sechellia* remained significantly higher than both *D. simulans* and the hybrids, however, it was no longer significantly greater than *D. melanogaster* (χ^2 test, 1 df, $P = 0.02996$, 0.0001467, and 0.1891, respectively). In the comparisons between species at each developmental transition, only the L to EP transition remained significantly greater in *D. sechellia* as compared to the other two species or the hybrids ($P < 0.001$). Figure 5.1A has been redrawn using the values obtained from the simulated, increased variance, *D. sechellia* data, and is presented as Figure 5.1B.

Between-species divergence in the context of ontogeny

The observed correlation between conservation of morphological features and nucleotide coding sequences during earlier stages of ontogeny (e.g., Davis et al. 2005; Cutter and Ward 2005; Artieri et al. 2009) may suggest that genes expressed during earlier stages of ontogeny should also be more conserved in terms of overall expression level in comparison to genes expressed at later stages. Therefore we compared the number of genes that were differentially expressed at each of the four sampled stages in pairwise comparisons among those genes detectibly expressed in all three pure species (2,253 genes) (Figure 5.2). We find that in those pairwise comparisons between species in which the number of differentially expressed genes varied significantly between sampled stages (*D. simulans* vs. *D. melanogaster*, and *D. simulans* vs. *D. sechellia*), fewer genes were differentially expressed at the earlier stages (χ^2 test, 1 df, $P < 0.001$). In no case were there significantly fewer genes differentially expressed at a later developmental stage in comparison to an earlier one ($P > 0.05$). Because *D. simulans* and *D. sechellia* form a clade excluding *D. melanogaster* (Kliman et al. 2000; Tamura et al. 2004), we expected that they would show fewer differentially expressed genes than the other two possible pairwise comparisons. This is the case in all situations in which the difference between comparisons is statistically significant for the 3rd instar larval, late pupation, and adult stages (Figure 5.2). However, in the case of the early pupal stage, while there are fewer genes differentially expressed in the comparison between *D. simulans* and *D. sechellia* than the comparison between *D. melanogaster* and *D. sechellia*, there are significantly more genes differentially expressed than the comparison between *D. melanogaster* and *D. simulans* ($P < 0.001$ in all cases). Despite sharing the same evolutionary distance from *D. melanogaster*, we found significant asymmetry in the number of genes differentially expressed in comparisons between *D. melanogaster* and *D. simulans* or *D. sechellia* during the 3rd instar larval and early pupation stages. More specifically, we noted that there are fewer genes differentially expressed between *D. melanogaster* and *D. simulans* as compared to *D. melanogaster* and *D. sechellia* in both cases ($P < 0.001$) suggesting that *D. simulans* and *D. sechellia* have experienced different evolutionary pressures in earlier developmental stages.

Previous studies have observed that particular classes of genes may more likely to diverge in expression level between species, such as those that are sex-biased in expression (Meiklejohn 2003). Therefore we assessed whether genes showing sex-bias in expression level (i.e., greater level of expression in one sex as compared to the other) were more likely to be differentially expressed between species. We used the data of Hambuch and Parsch (2005), which pooled together the results of several studies of sex-specific expression conducted in *D. melanogaster*, in order to determine whether significant differences existed in the proportions of differentially expressed male-biased (MBG), female-biased (FBG), and unbiased genes (UBG). In the case of the comparisons between *D. melanogaster* and either *D. sechellia* or *D. simulans*, we find that MBGs are signifcantly over-represented among those genes that are significantly differentially expressed during the 3rd instar larval stage ((χ^2 test, 1 df, $P = 0.0222$, and 0.0329,

respectively). We find no statistically significant differences in the proportions of MBGs, FBGs, or UBGs among those that are significantly differentially expressed between species after correction for multiple tests in any other comparisons; however, there is a non-significant tendency for a greater proportion of MBGs to be represented among significantly differentially expressed genes as compared to those that are not significantly differentially expressed (χ^2 test, 1 df, $P > 0.05$) (Supplementary Table 4).

Hybrid expression patterns in the context of ontogeny

As was the case for the comparison between the pure species, we compared the number of genes that were differentially expressed in pairwise comparisons between the hybrids and their parental species at each stage among those genes detectibly expressed in all three, during all four stages (2,052 genes) (Figure 5.3). We observed no significant differences among stages in the number of genes differentially expressed between *D. simulans* or the hybrid (χ^2 test, 3 df, $P = 0.1322$). However, in the case of the comparison between *D. sechellia* and the hybrid, both the early and late pupal stages showed significantly fewer differentially expressed genes than either the 3rd instar larval or adult stages (χ^2 test, 1 df, $P = 0$). The number of genes differing significantly in expression level between parental species and the hybrids was significantly different between the two parents (i.e., *D. simulans* vs. the hybrids as compared to *D. sechellia* vs. the hybrids) ($P < 0.001$): the 3rd instar larval and adult stages showed a greater number of differentially expressed genes in *D. sechellia*, whereas the early- and late pupal stages showed the opposite pattern. Finally, in all stages where a significant asymmetry was observed in the number of genes that are expressed at a higher level in the parental species as compared to the hybrid, the hybrid showed a lower number of genes with higher expression levels, as is expected from previous studies that have revealed a general trend towards underexpression in the hybrids as compared to their same-sexed parental species (e.g., Haerty and Singh 2006, Ortíz-Barrientos et al. 2007; Artieri et al. 2007).

Numerous studies have highlighted the general phenomenon of interspecific hybrid misexpression, defined as genes that are either significantly under- or over-expressed in hybrids as compared to both of their parents (Michalak and Noor 2003); however, these studies have generally focused on expression patterns derived from a single stage (see Ortiz-Barrientos et al. 2007 for review). The sole exception is the the study of Moehring et al. (2007), that sampled a small number of genes (48) involved in spermatogenesis in hybrids of species of the *D. simulans* clade during the late larval and adult stages and noted that fewer genes were misexpressed during the larval stage (3) as compared to the adult stage (7). In order to gain a better understanding of how hybrid misexpression relates to development we compared the expression in the *D. sim* × *D. sec* hybrid males to male expression patterns in the parental species (Figures 5.4 and 5.5, Table 5.1). As indicated above, a number of authors (e.g., Reiland and Noor 2002, Ortíz-Barrientos et al. 2007) have suggested that the widespread patterns of misexpression

observed in adult flies may result from the downstream cascading effects of a smaller number of developmentally important genes that are misexpressed at early ontogenic stages. However, contrary to the expectations of a cascading model of hybrid misexpression, we observed that the 3rd instar larval stage shows the highest number of misexpressed genes (303), while the fewest number of misexpressed genes is observed during the late pupal stage (24), such that the pattern of proportion of misexpressed genes, from fewest to most, is: late pupal < early pupal < adult < 3rd instar larval (the difference in the proportion of genes that are misexpressed is statistically significant among all pairwise comparisons between stages; χ^2 test, 1 df, $P < 0.001$) (Table 5.1). Again, supporting the results of previous studies (e.g., Haerty and Singh 2006, Ortíz-Barrientos et al. 2007; Artieri et al. 2007) we find that for all stages in which the difference is statistically significant (3rd instar larval, early pupal, and adult), a greater proportion of genes are underexpressed in the hybrids as compared to overexpressed (χ^2 goodness of fit test, 1 df, $P < 0.001$).

If interactions among loci derived from each parent in the hybrids were perfectly additive, we would expect the hybrid to display a phenotype intermediate between that of both parents. However, numerous studies have shown that additivity is often not the rule (e.g., Gibson et al. 2004; Ranz et al. 2004; Haerty et al. 2006), thus we compared the expression patterns of genes in the hybrids independently to each parent in order to determine whether the hybrids were more similar to one parent rather than the other. We performed an analysis of covariance (ANCOVA) on the regression of the $\log_2$(sample/reference) expression values of the *D. sim* × *D. sec* hybrids upon that of each of the parents (Supplementary Figure 5.3), to determine whether the regression of one parental species remained significant if its regression upon the other parent was held constant (ANCOVA formula: Hybrid Expression values ~ *D. sechellia* expression values * *D. simulans* expression values). In the case of the 3rd instar larval, early-, and late pupal stages, the interaction term was non-significant ($F_{1,2050} = 3.0599$, 1.8441, and 0.4118, $P =$ 0.0804, 0.1746, and 0.5211, respectively) indicating that expression levels in the hybrids are not significantly more correlated with one parent as compared to the other. In the case of the adult stage, however, we find that there is a significant difference between the correlations between the hybrid and each parent ($F_{1,2050} = 33.724$, $P = 7.347 \times 10^{-9}$), indicating that expression values are more similar between *D. simulans* and the hybrid (slope [m] = 0.8214, intercept [b] = -0.01168, $r^2 = 0.6477$) than *D. sechellia* and the hybrid (m = 0.7594, b = 0.01099, $r^2 = 0.6139$). Another method by which to assess whether one parent is more similar to the hybrids than the other is to ask how the hybrid resembles each parent in terms of the degree to which genes change in expression between developmental transitions (i.e., L to EP, EP to LP, and LP to A). Therefore we restricted our analysis only to those genes that varied significantly in expression level in both parental species and the hybrids at each developmental transition, and performed linear regressions followed by ANCOVA to determine if the hybrids are more similar to one parental species than the other (Supplementary Figure 5.4). We found that the hybrids

were significantly more correlated with one parent as compared to the other during the EP to LP and LP to A transitions, whereas the hybrid was not significantly more similar to either parent during the L to EP transition ($F_{298, 436, \text{and } 244} = 3.1602$, 19.007, and 112.72, P = 0.07647, 1.626×10^{-5}, $< 2.2 \times 10^{-16}$, for the L to EP, EP to LP, and LP to A transitions, respectively). In the case of the EP to LP transition, the degree to which genes change in expression level between transitions the hybrid is more significantly correlated with *D. sechellia* (m = 0.8963, $r^2 = 0.9219$) than *D. simulans* (m = 1.531, $r^2 = 0.8381$). However, during the LP to A transition, the hybrid is more significantly correlated to the *D. simulans* parent (m = 1.157, $r^2 = 0.6417$) as compared to the *D. sechellia* parent (m = 0.6630, $r^2 = 0.8011$).

Microarray validation by quantitative RT-PCR

Five genes from the list of those that were detectibly expressed in all 3 parental species as well as the hybrids were randomly selected for use in quantitative Reverse Transcriptase PCR (qRT-PCR) validation of the results of the microarray analysis (see Materials and Methods; Supplementary Table 3). We pooled estimates of fold change expression differences between species/hybrids at each stage, and between developmental transitions within species, and found a significant correlation between those estimated from qRT-PCR and microarray analysis (Spearman's rank correlation test [in all cases], $\rho = 0.4998$, $P = 6.135 \times 10^{-7}$) (Supplementary Figure 5.5; Supplementary Table 5). Given the potential for hybridization bias derived from the use of a *D. melanogaster* cDNA spotted microarray to measure expression levels in other species, we also tested if the correlation differed between expression level fold change estimates between developmental stages within species as compared to estimates derived from same-stage comparisons between species. Limiting our analysis only to the case of within species comparisons, the correlation improved ($\rho = 0.8036$, $P = 2.069 \times 10^{-7}$), while the correlation derived exclusively from between species comparisons became non-significant ($\rho = 0.1592$, P = 0.2548), suggesting that single-species microarrays may impose substantial bias upon estimates of expression levels (comparing within species presumably has the effect of cancelling out a significant amount of hybridization bias) (Supplementary Figure 5.5). Manual inspection of the data revealed a slight but non-significant tendency for qRT-PCR estimates of the fold change difference between species to be more likely of opposite sign (e.g., an increase in expression in qPCR estimates as compared to a decrease in expression in microarray estimates) in comparisons between *D. melanogaster* or hybrids and *D. sechellia* (13/27) when compared to those involving *D. melanogaster* or the hybrids and *D. simulans* (4/18) (χ^2 test, 1 df, P = 0.2269). Therefore, we tested whether the correlation between qRT-PCR and microarray estimates of fold change difference in comparisons between species differed when we limited our correlations of expression level fold change estimates to those involving *D. simulans* but not *D. sechellia*, and vice versa. We found that the

between-species correlations that excluded *D. sechellia* were statistically significant (ρ = 0.5232, P = 0.02736), whereas those excluding *D. simulans* were not (ρ = 0.02442, P = 0.9030). Furthermore, when limiting our analysis to within species estimates of fold change expression difference between stages, we find that the coefficients of correlation for *D. melanogaster*, *D. simulans*, and the *D. sim* × *D. sec* hybrids are all greater than 0.80 (P = 0.02381, 1.929×10^{-6}, and 0.05833, respectively), whereas *D. sechellia* shows a coefficient of correlation of 0.5944 (P = 0.04426), suggesting that one of the two methods of estimating expression differences, qRT-PCR or microarrays, is incorrectly assessing expression levels in *D. sechellia*.

Another, albeit crude, method through which to assess the potential for hybridization bias confounding estimates of expression level is to test for a correlation between sequence divergence between species and absolute fold change in expression level as estimated from microarrays (Artieri et al. 2007). If differences in expression level resulted entirely from differences in binding efficiency due to sequence divergence, then we should expect a positive correlation between expression and sequence divergence. We find no significant positive correlation after Bonferroni correction between our standard pairwise estimates of sequence divergence (d_N, d_S, and d_N/d_S) and pairwise comparisons of absolute significant fold changes in expression divergence (P > 0.05).

5.5 Discussion

Consideration of Hybridization Bias of Interspecific Transcripts on the *D. melanogaster* Microarray

A number of studies in the field of speciation have used single species microarrays, both cDNA (Ranz et al. 2003, 2004; Haerty and Singh 2006; Artieri et al. 2007; Moehring et al. 2007) and oligonucleotide (Michalak and Noor 2003; Malone et al. 2006, 2007; Barbash and Lorrigan 2007), in interspecific comparisons of expression levels. However, it has been well established that sequence divergence between the sample being hybridized and the spotted probes leads to biased estimates of expression divergence if appropriately conservative significance thresholds are not applied during analysis (Gilad et al. 2005). Some of interspecific microarray hybridization studies cited above employed strategies in order to rule out the effect of sequence bias, such as correcting expression divergence estimates by the baseline hybridization ratio observed when equal concentrations of two species' sheared genomic DNA are applied to the array (Malone et al. 2007), or by testing for a potential correlation between sequence and expression divergence (Artieri et al. 2007). However, to our knowledge, with the exception of the study of Michalak and Noor (2003; 2004) which confirmed that 5 genes in their study were underexpressed in hybrids, none of these studies has attempted to validate the results of their microarray based transcriptional profiles using qRT-PCR, despite such validation being a common protocol in other fields employing whole

transcriptome gene expression analysis (Morey et al. 2006). The correlation coefficients that we observed using our entire dataset for the 5 genes surveyed by qRT-PCR (ρ = 0.4998), or in between species comparisons excluding *D. sechellia*, (ρ = 0.5232), are in line with previously published results that have intended to validate intraspecific hybridizations, (ρ = 0.633 – 0.748) (Morey et al. 2006). The slightly reduced correlation coefficients observed in our own analysis may reflect the effect of sequence divergence on hybridization.

Excluding *D. sechellia*, we also observed high coefficients of correlation between fold changes in expression level as estimated from qRT-PCR and microarrays for transitions within species ($\rho > 0.80$). The potential for hybridization biases due to sequence divergence between mRNA and spotted probes should be limited in the case of within-species comparison of expression levels as any such biases would be expected to be equally present in each sample and thus cancel out (Rifkin et al. 2003). Therefore, the improved correlation coefficients observed for within- as compared to between species comparisons likely indicates that hybridization bias resulting from interspecific transcripts is not trivial, and results should be interpreted with caution. However, previous analyses have shown that using appropriate statistical thresholds in terms of which genes are considered significantly differentially expressed can lead to accurate assessment of expression differences on a single species array (Gilad et al. 2005). We chose a 1.5 fold threshold difference in expression level as this threshold was shown to provide near 100% specificity (albeit at a cost in sensitivity) to accurately measuring significant expression differences between human and orangutan mRNA samples hybridized on a human microarrays, which differ in nucleotide sequence by approximately 3% (*D. melanogaster* and *D. simulans*/*D sechellia* differ ~3%; Heger and Ponting 2007).

In the case of estimates of fold change expression differences involving comparisons with *D. sechellia*, the results of microarray analysis would appear to be incongruent with those of qRT-PCR. As noted above, coefficients of correlation between the two techniques derived from within species comparisons were high for all species except for *D. sechellia* (ρ = 0.5944) suggesting that at least some of the *D. sechellia* cDNA samples used to perform qRT-PCR were either contaminated or derived from degraded mRNA. Analysis of the data pooled for all sampled stages shows that pairwise coefficients of correlation among $\log_2$(sample/reference) estimates of expression level between the three pure species match phylogenetic expectations (i.e., ρ = 0.7213 and 0.7695 for the correlation between *D. melanogaster* and *D. sechellia* / *D. simulans*, and ρ = 0.7975 for the correlation between *D. sechellia* and *D. simulans*; $P < 2.2 \times 10^{-16}$ in all cases). Thus, given that pairwise nucleotide divergence estimates between *D. sechellia* and *D. melanogaster* are similar to those of *D. melanogaster* and *D. simulans*, (Stark et al. 2007) it seems more parsimonious to assume that the *D. sechellia* samples used for qRT-PCR were inadequate, though confirmation of this hypothesis will require further testing.

Within-species variation in expression levels over ontogeny

Our estimates of the percentage of genes that vary significantly in expression level over the course of ontogeny in the three pure species and the hybrids (~60-80%) are similar to those reported in a previous analysis of gene expression over the entire course of ontogeny in *D. melanogaster* (~86%; Arbeitman et al. 2002). The reduction in the proportion of genes varying significantly in expression level between stages in our study is most likely a result of our having sampled only the latter portion of development, whereas the aforementioned analysis also sampled many stages of embryogenesis. The significant increase in the proportion of developmentally modulated genes in *D. sechellia* is striking (Figure 5.1A), especially given the similarity of numbers estimated in the other two species and the hybrids. However, as noted above, a potential, non-evolutionary explanation for this observation could be a systematic bias towards low between-replicate variance in *D. sechellia* as compared to the other samples, thus improving our statistical power to detect significant differences between samples. Such a bias was observed (Figure 5.1B), and our corrected estimates of the number of modulated genes in *D. sechellia* reveal that the effects of this bias are significant (i.e., much of the elevated signal of developmental modulation in *D. sechellia* disappears; Figure 5.1 B). Regardless, the general pattern of an elevated number of developmentally modulated genes in *D. sechellia* remains significant among all comparisons for genes varying significantly in expression level during the L to EP transition, suggesting that *D. sechellia* may have undergone lineage specific divergence in terms of its developmental expression profiles (see below). It is also interesting to note that the observed reduction in between replicate variance observed in *D. sechellia* is consistent with previous population genetics studies of nucleotide diversity in this species that have found a significantly reduced level of within species polymorphism relative to *D. simulans* (e.g., Hey and Kliman 1993; Kliman et al. 2000). Numerous lines of evidence suggest that *D. sechellia* arose from a relatively recent island colonization event, perhaps having gone through a severe bottleneck, and having maintained low effective population sizes since, which may explain the overall reduction in expression estimate variability observed in this species, (Kliman et al. 2000).

Between-species divergence in the context of ontogeny

The results of our between species comparisons of the proportion of genes that are differentially expressed between the three pure *Drosophila* species generally support what has already been observed in the context of both morphology and nucleotide sequences: when comparisons between stages are statistically significant, earlier stages are more conserved at the level of gene expression than later stages (Figure 5.2). In no comparison were significantly fewer genes differentially expressed between species at later stages as compared to earlier stages. Thus it would appear that Von Baer's (1828) classic observation that earlier stages of ontogeny are more conserved than later stages applies at both nucleotide (e.g., Davis et al. 2005; Cutter and Ward 2005; Artieri et al. 2009) and

transcriptome levels. The number of genes differentially expressed in our pairwise comparisons support phylogenetic expectations in the case of the 3rd instar larval, late pupal, and adult stages (i.e., the more closely related species, *D. sechellia* and *D. simulans* show fewer significantly differentially expressed genes than more distant evolutionary comparisons when these differences are statistically significant), however, such is not the case during the early pupal stage, where the comparison between *D. melanogaster* and *D. simulans* shows the fewest significantly differentially expressed genes (Figure 5.2). This may suggest that, as part of its adaptation to its host plant, *Morinda citrifolia*, *D. sechellia* may have been exposed to unique selective pressures that have altered particular aspects of its larval or early pupal development. This notion is supported by previous studies that have examined developmental phenotypes in the *D. melanogaster* group (e.g., Sucena and Stern 2000; Lott et al. 2007; Markow et al. 2008) and that have found evidence of altered developmental phenotypes specifically in *D. sechellia* relative to other species. Interestingly, we found little evidence of significant overrepresentation of MBGs among genes that were differentially expressed between species (Supplementary Table 4), as has been previously reported in the case of the comparisons between species of the *D. melanogaster* group (Meiklejohn et al. 2003; Zhang et al. 2007). However, it should be noted that both aforementioned studies used experimental designs that should be more sensitive to detecting interspecific expression divergence (i.e., direct interspecific competitive hybridization in the former case, and species-specific microarrays in the latter), and thus our failure to find a significant effect of sex-bias may simply reflect reduced sensitivity of our reference-based experimental design (Churchill et al. 2002). It is also worth noting that while non-significant, most comparisons did show an over-representation of MBGs among those that varied significantly.

Hybrid expression patterns in the context of ontogeny

Studies in both the fields of evo-devo as well as speciation have long used interspecific hybrids in order to study the genetic architecture underlying phenotypic divergence between species (reviewed in Coyne and Orr 2004). However, while evo-devo and developmental genetics have long been interested in the mechanisms of gene regulation, it is only in the last decade that the field of speciation research has recognized that the wealth of interactions involved in the process of transcriptional regulation provide extensive opportunity for the evolution of Bateson-Dobzhansky-Muller incompatibilities, which arise from the independent divergence of interacting factors in separate lineages that fail to complement one another when brought together in hybrids (Bateson 1909; Dobzhansky 1936; Muller 1942). This recognition has led to a number of studies involving genome-scale transcriptional profiling in interspecific hybrids (reviewed in Ortíz-Barrientos et al. 2007), which have revealed varying degrees of gene misexpression in hybrids relative to their parental species (up to 69% of genes surveyed; Ranz et al. 2004). While a proportion of such misexpression certainly results from allometric effects

of developmental anomalies in hybrids, those of *D. melanogaster* and *D. simulans* fail to develop a germline, for instance (Sturtevant 1920), a significant proportion of these genes are legitimately underexpressed (typically) in hybrids, especially in cases of hybrids that do not show significant allometric defects in development, such as is the case in F1 hybrids between *D. simulans* (♀) × *D. sechellia* (♂) (Civetta and Singh 1998). However, given that loci involved in Bateson-Dobzhansky-Muller incompatibilities typically map to localized and specific regions of the genome (e.g., Orr et al. 2007), it appears unlikely that the widespread phenomenon of interspecific hybrid misexpression results from the incompatible divergence of *cis*-regulatory factors at such a large number of loci. Rather it seems more plausible that large scale-patterns of misregulation observed in interspecific hybrids result from divergence of a smaller number of loci having widespread effects in *trans* (Noor 2005).

Our study does not support the suggestion that these *trans* acting factors are derived from the cascading effects of a smaller number of genes that are significantly misexpressed at earlier stages of development (e.g., Reiland and Noor 2002; Ortíz-Barrientos et al. 2007). Rather it would appear that there is considerable stage-specific autonomy of regulatory 'breakdown', with the intermediate stages of our sampled ontogenic interval having the smallest proportion of genes significantly misexpressed (Figure 5.4, 5.5; Table 5.1). Furthermore, the 3rd instar larval stage is the stage during which the greatest proportion of genes are significantly misexpressed in the hybrids. Two non-mutually exclusive hypotheses may account for an elevated proportion of misexpressed genes during this stage. Firstly, the larval stage is characterized by a high rate of growth (approximately 200 fold increase in wet mass from a newly hatched larva to full grown 3rd instar; Siard et al. 1991), which is associated with a rapid increase in transcription of total mRNA and translation of proteins (Viccario et al. 2008). Slight heterochronic changes in hybrid development (e.g., later activation of transcriptional machinery) may manifest themselves as widespread under- or even overexpression of larval genes. Secondly, previous studies have suggested that D. *sechellia* has also been subject to divergence in embryonic and larval ontogeny, perhaps as a result of its specialization to its host plant *M. citrifolia* (Sucena and Stern 2000; Lott et al. 2007). It is possible that while this divergence may not manifest itself as in terms of expression level differences in the parental species (Figure 5.3, Supplementary Figure 5.3), perhaps due to developmental systems drift (True and Haag 2001), it could be revealed through the creation of incompatibilities in the hybrid, generating an elevated proportion of incompatibilities during this stage.

A classic study testing the vulnerability of various stages of *Drosophila* ontogeny to induced mortality when exposed to X-rays found that susceptibility is highest during pupation, suggesting that this stage is particularly sensitive to deleterious perturbation (Woskressensky 1928). Such observation is particularly interesting in the context of Raff's (1994, 1996) 'developmental hourglass' hypothesis, which suggests that particular developmental stages, especially those involving morphogenesis, involve a greater degree

of integration between regulatory circuits, and thus may be more resistant to evolutionary divergence than preceding or subsequent stages. While it is important to remember that we only sampled the latter portion of *Drosophila* development, and that we remain ignorant about what patterns of misexpression may be occurring during embryogenesis and early larval periods, our observation of a significantly reduced number of misexpressed genes during the two sampled pupal stages as compared to the 3rd instar larval or adult stages, suggests that the mechanisms underlying gene regulation during these stages may be more conserved (Table 5.1). It is interesting, however, that if one restricts their analysis to genes expressed in the hybrids that are significantly differentially expressed between males of *D. simulans* and *D. sechellia*, while they are significantly more likely to be expressed at the *D. simulans* level in the hybrids during the 3rd instar larval and adult stages, they are more likely to be expressed at *D. sechellia* levels during the two sampled time points during the pupal stage (Figures 5.4, 5.5, Table 5.1). One may assume that expression levels would generally show an overall dominance in hybrid males in the direction of the parent from which it inherits its X chromosome (in this case *D. simulans*), assuming, of course, that some significant number of regulatory loci are harbored on the X. While this may be the case in the early and latest stages, our results suggest that while the ability of regulatory factors to interact is more conserved during the pupal stages, significant divergence has occurred between the two parental species that manifests itself dominantly with regards to the *D. sechellia* parent in the hybrids during these stages. This hypothesis is supported by our observation that the degree to which genes vary in expression level between developmental transitions in hybrids is significantly more similar to the *D. sechellia* parent during the EP to LP transition (Supplementary Figure 5.4)

Conclusions

In summary, our comparative analysis of transcriptional patterns over the course of ontogeny among species and hybrids of the *D. melanogaster* group has revealed the following major results: 1) genes expressed at earlier stages of development tend to be more conserved among species in terms of expression level than those expressed at later stages, 2) the number of genes differentially expressed between stages support phylogenetic expectations (i.e., are fewer in comparisons between *D. simulans* and *D. sechellia*) for all stages except the early pupal stage, 3) there is considerable stage-specific autonomy of regulatory breakdown, which does not support a cascading model explaining hybrid misexpression, 4) the factors underlying gene expression appear to be more conserved during the pupal stage as compared to earlier of later stages, and finally 5) that genes that vary significantly in expression level over development appear to be more conserved in their coding sequences in comparison to genes that are stably expressed (see Appendix § 5.6). Our findings have implications for the fields of both evo-devo and speciation. Firstly, they support the extension of Von Baer's (1828) 'third law', or the more modern developmental hourglass hypothesis, to the level of the

transcriptome, further supporting previous non-developmentally-based observations suggesting that similar forces may act to limit both gene expression levels and coding sequence divergence (e.g., Artieri et al. 2007). Secondly, while it has already been remarked that the widespread misexpression of genes observed in interspecific hybrids is unlikely to be the result of equally widespread divergence of *cis* regulatory elements (e.g., Noor et al. 2005; Ortíz-Barrientos et al. 2007) our results suggest that it is unlikely that the *trans* factors underlying misexpression result from developmental cascading effects. Rather, it appears more likely that regulatory factors (e.g., proteins, mRNAs, etc.) experience stage-specific, autonomous incompatibilities, leading to similarly stage-specific patterns of misexpression. Several of the so-called 'speciation' genes (i.e., loci that contribute to hybrid dysfunctions such as sterility or inviability) that have been identified so far are predicted to have transcription factor activity, and regulate expression of downstream genes in *trans* (see Michalak and Noor 2004). The findings presented here suggest that a more complete understanding of stage-specific gene regulatory networks, such that we can identify those nodes that may ultimately control the suite of genes identified as misexpressed in hybrids may be a fruitful approach to identifying new loci underlying both developmental evolution, reproductive isolation, and ultimately speciation.

5.6 Appendix: Analysis of Rates of Coding Sequence Divergence over Sampled Developmental Stages.

Artieri et al. (2009) found a significant increase in the mean rate of divergence among genes expressed in subsequent stages of *D. melanogaster* ontogeny, classified by the stage at which they showed their highest proportion of representation in stage-specific EST libraries. However, their study was unable to separate genes expressed in the larval stage from those expressed during the pupal stage due to the lack of larval and pupal specific EST libraries. In order to address the dynamics of gene divergence among genes expressed during these stages, we classified genes in the three pure species and the hybrids into our 4 sampled developmental stages based on the stage at which a gene showed its highest level of expression using a series of arbitrarily chosen specificity thresholds (see Materials and Methods), and obtained gene divergence information (d_N, d_S, and d_N/d_S) for all genes in our dataset that were represented in the *D. melanogaster* group set of *Drosophila* 12 Genomes Consortium (2007) data (1,334 genes). While the comparisons between genes classified into specific stages using no specificity threshold revealed statistically significant differences (see below; Supplementary Table 6), very few comparisons remained significant even at low specificity thresholds, likely owing to the much reduced sizes of the datasets. Consequently, we present only the results of the analysis using no specificity threshold, though all results of the analysis performed using a greater than 0.25, or 0.5 fold threshold can be found in Supplementary Table 6. Like Artieri et al. (2009), in *D. melanogaster*, we find that when comparisons among divergence rates (d_N and d_N/d_S) between stages are statistically significant, later stages diverge more rapidly than earlier stages (Kruskal-Wallis rank sum test, $P < 0.05$ for the comparisons between either the late pupal or adult stages with the 3rd instar larval stage); the late pupal stage also diverges more rapidly than the early pupal stage in terms of d_N/d_S ($P = 0.049$) (Supplementary Table 6). In terms of d_S, the only significant comparison indicated that the synonymous rate of substitution was lower in the 3rd instar larva stage as compared to the early pupal stage, possibly lending weak support to the findings of previous studies suggesting that codon usage bias is highest during the larval stage (Vicario et al. 2008). In *D. sechellia*, the only statistically significant comparison in terms of d_N and d_N/d_S indicates that the adult stage is diverging more rapidly than the early pupal stage ($P < 2.2 \times 10^{-16}$); the adult stage is also diverging more rapidly than the late pupal stage in d_N ($P = 0.04$). With regard to d_S, the late pupal stage appears to be more conserved than the 3rd instar larval and early pupal stages. Interestingly, all comparisons between stages in *D. simulans* were non-significant. Finally, in the case of the hybrid, we found that the late pupal stage diverges more rapidly (d_N and d_N/d_S) than the other three stages, while all other comparisons were non-significant, with the exception of the early pupal stage, which is more conserved than the late pupal stages with regards to d_S (Supplementary Table 6).

Differences between species in the patterns of divergence rates over ontogeny raises the possibility that the stage of highest gene expression may vary significantly between species, especially in comparisons over large evolutionary distances (Artieri et al. 2009). In order to address this, we compared the proportion of genes that retained the same stage of highest expression in comparisons between *D. melanogaster* and *D. simulans*, as well as *D. sechellia* and *D. simulans* at no specificity threshold (1,334 genes), and a greater than 0.25 or 0.5 fold expression level threshold (212 and 93 genes, respectively). Using no specificity threshold, we find that 53% of genes (708) retain the same stage of highest level of expression in *D. melanogaster* and *D. simulans*, while 55% of genes (732) retain the same stage of highest level of expression in *D. sechellia* and *D. simulans*. Using a greater than 0.25 fold threshold of expression, these numbers become 63% (134 genes) and 76% (161 genes) respectively, a statistically significant increase in the case of the *D. sechellia/D. simulans* comparison (χ^2 test, 1 df, P = 0.00433). Finally when comparing patterns classified at a 0.5 fold or greater specificity threshold, 98% of genes (91/93) retained the same highest stage of expression in both pairwise comparisons, which is a statistically significant increase in conservation of stage of highest expression level as compared to using no specificity threshold in both comparisons (χ^2 test, 1 df, P < 0.001). These results suggest that the more specific a gene's expression is to a given stage, the more likely it is to retain such specificity in evolutionary comparisons.

In addition to evolutionary patterns relating to the stage at which genes show their highest level of expression, genes that vary significantly in expression level during development may be expected to be subject to different evolutionary forces as compared to those whose expression remains stable. We find that in all 3 pure species as well as the hybrids, genes that vary significantly in expression level diverge less rapidly in terms of d_N than those genes that vary significantly (permuted Kruskal-Wallis rank sum test, P < 0.05). This pattern also holds for d_S (P < 0.001) except in the case of *D. sechellia* (P = 1.00). Mirroring this pattern, the distribution of values of d_N/d_S is significantly greater in genes that vary significantly over development as compared to those that do not vary only in *D. sechellia* (p = 0.0102); there are no statistically significant differences in the other 3 comparisons.

Our results generally support those of Artieri et al. (2009) in that in *D. melanogaster*, when the differences are statistically significant, later stages diverge more rapidly than earlier stages (Supplementary Table 6). This pattern also holds in *D. sechellia* in that statistically significant differences in evolutionary rates are in the direction of more rapid divergence in later stages. In the case of the *D.sim* × *D.sec* hybrids, the late pupal stage appears to be the most rapidly diverging in terms of both d_N and d_N/d_S. Interestingly, significantly more genes expressed at their highest level in the adult stage of *D. sechellia* are expressed at their highest level during the late pupal stage in hybrids in comparison to the 3rd instar larval or early pupal stages (χ^2 test, 1 df, P < 0.001). Furthermore, the proportion of genes expressed at their highest level in adults in

D. sechellia was not significantly different in comparison between genes expressed at their highest level in the late pupal or adult stage of the hybrids, perhaps indicating that a significant portion of genes expressed at an earlier stage in the hybrids as compared to *D. sechellia*, thus leading to a signal of elevated d_N and d_N/d_S in the late pupal stage. However, without a larger dataset of genes such possibilities must be considered purely speculative.

Another significant result of Artieri et al.'s (2009) analysis was the observation of a positive correlation between the rate of divergence of genes and the degree to which those genes were specific in the stage at which they were expressed. We were unable to test the results of their analysis in our interspecific comparisons due to the reduced sample-sizes of genes classified into specific stages at even low (i.e., greater than 0.25 higher expression than any other stage) specificity thresholds. However, the ability to repeat such comparisons using a larger data set would be useful, given our observation that genes that are more specific in expression to a given developmental stage are more likely to be conserved in terms of stage of highest expression level between species, and thus would likely provide a more adequate form of comparison of divergence rates over ontogeny between species. Failure to apply adequate specificity thresholds will likely add noise to the analysis resulting from normal intraspecific or intraindividual variance in expression levels, which may explain why no significant differences were observed in comparisons between stages in *D. simulans*. However, despite the variability observed in the rates of divergence of genes expressed at their highest level in different stages of ontogeny, we observed a consistent pattern in all three species and the hybrids indicating that genes that vary significantly in expression level over development diverge less rapidly in terms of d_N than do genes that do not vary significantly (Table 5.2). This pattern also holds for d_S, but not d_N/d_S in all species/hybrids, with the exception of *D. sechellia* where the difference in terms of d_S between genes that do or do not vary significantly in expression level over development is non-significant, but the latter have an elevated d_N/d_S. Given that in *D. melanogaster*, *D. simulans*, and the hybrids, both the non-synonymous and synonymous rates of substitution, but not their ratio, are elevated in invariant genes, the most likely explanation is relaxation of selective constraint upon genes that are not modulated in expression level over development. The elevated d_N/d_S observed in *D. sechellia* among genes that do not vary over development may suggest the action of selection; however, given our observation of a significantly reduced between replicate variance in this species, likely leading to an overestimate of the proportion of genes that vary over the course of development, we cannot rule out the possibility that *D. sechellia's* unique pattern is simply an artifact of low within-species expression diversity.

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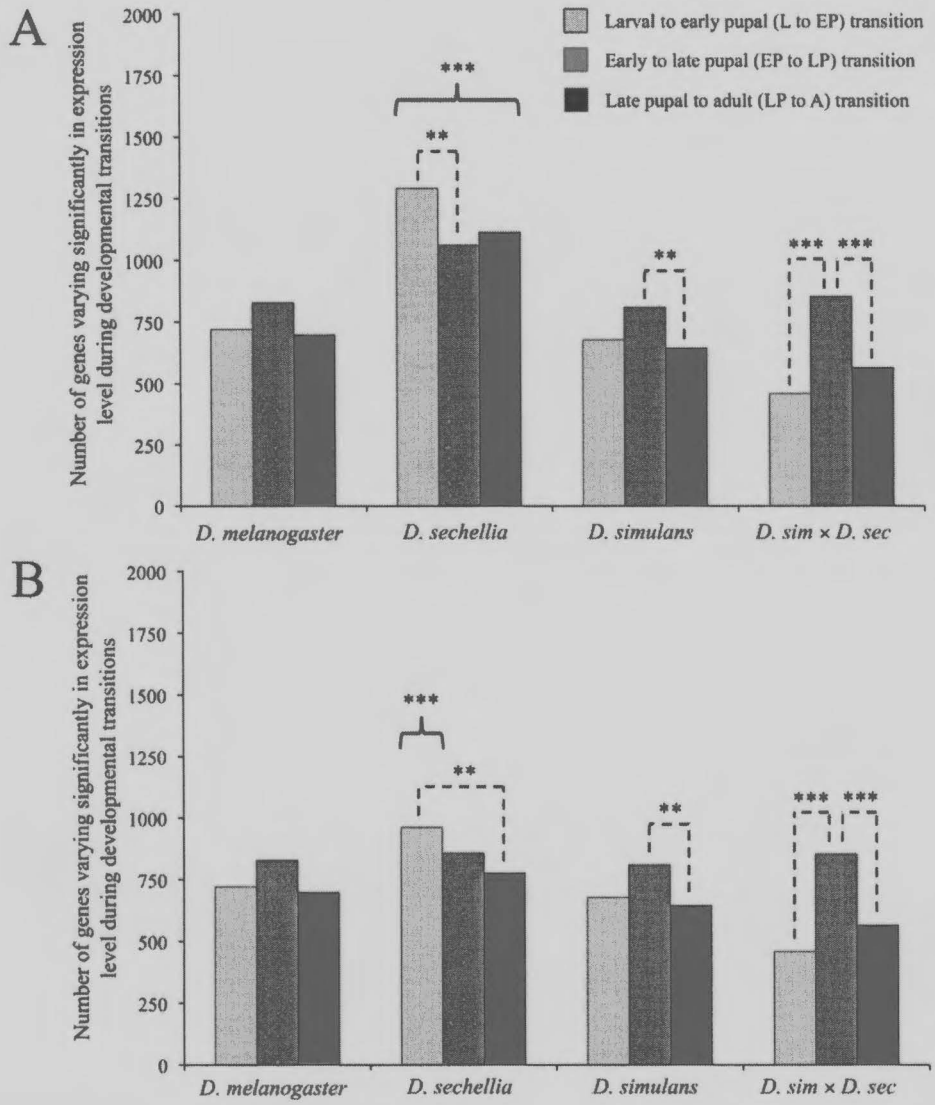


Figure 5.1: (**A**) Number of genes varying significantly in expression level in pairwise comparisons between each sequential developmental stage. The number of genes varying (out of 2,006 genes detectably expressed in all 3 species and the hybrid) is shown for each pairwise comparison (from left to right): 3rd instar larva to early pupa, early to late pupa, and late pupa to adult. Significantly more genes vary in expression level in each pairwise comparison in *D. sechellia* as compared to the other two species and the hybrid (indicated by the horizontal brace). All significant pairwise comparisions between transitions within species are indicated by dashed lines. P values of Bonferroni corrected χ^2 tests (1 df) are abbreviated as follows: ***, $P < 0.001$; **, $P < 0.01$. (**B**) Same figure as above, redrawn using the data derived from simulated *D. sechellia* mean expression values with variances scaled to *D. simulans* levels (see Results).

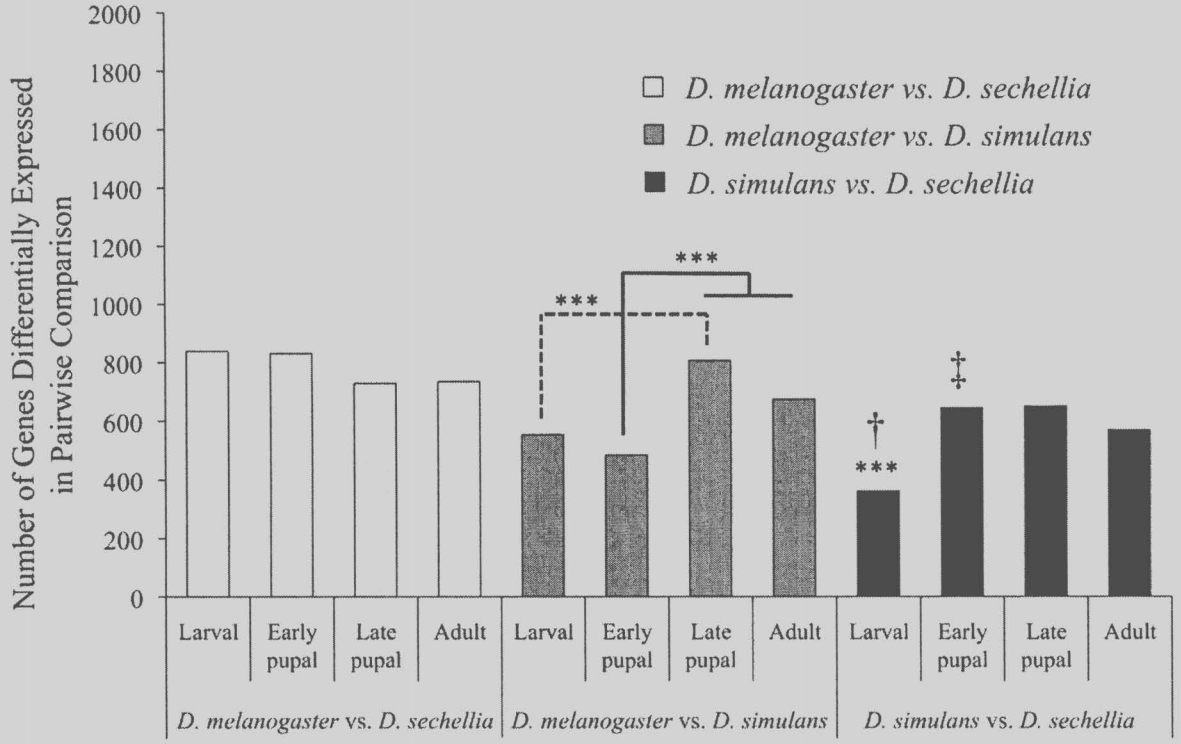


Figure 5.2: Number of genes significantly differentially expressed in pairwise comparisons between each of the three pure *Drosophila* species at each of the four sampled stages (out of 2,253 genes). There are fewer genes differentially expressed in the 3rd instar larva as compared to the late pupal stage in the comparison between *D. melanogaster* and *D. simulans* ($P < 0.001$) as indicated by the dashed line. Similarly, there are fewer genes differentially expressed in the early pupal stage of the same comparison than the late pupal or the adult as indicated by the solid line ($P < 0.001$). In the comparison between *D. simulans* and *D. sechellia* there are fewer genes differentially expressed at the 3rd instar larva stage than any other stage as indicated by the three asterisks (***) ($P < 0.001$). All other between stage comparisons within the pairwise comparisons were not statistically significant. There were fewer genes that were differentially expressed between *D. simulans* and *D. sechellia* at the 3rd instar larval stage than the other two more phylogenetically distant comparisons (indicated by the dagger [†]). The number of genes that were significantly differentially expressed differed in all three comparisons involving the early pupal stage; however, the *D. simulans* vs. *D. sechellia* comparison was not the lowest (indicated by the double-dagger[‡]).

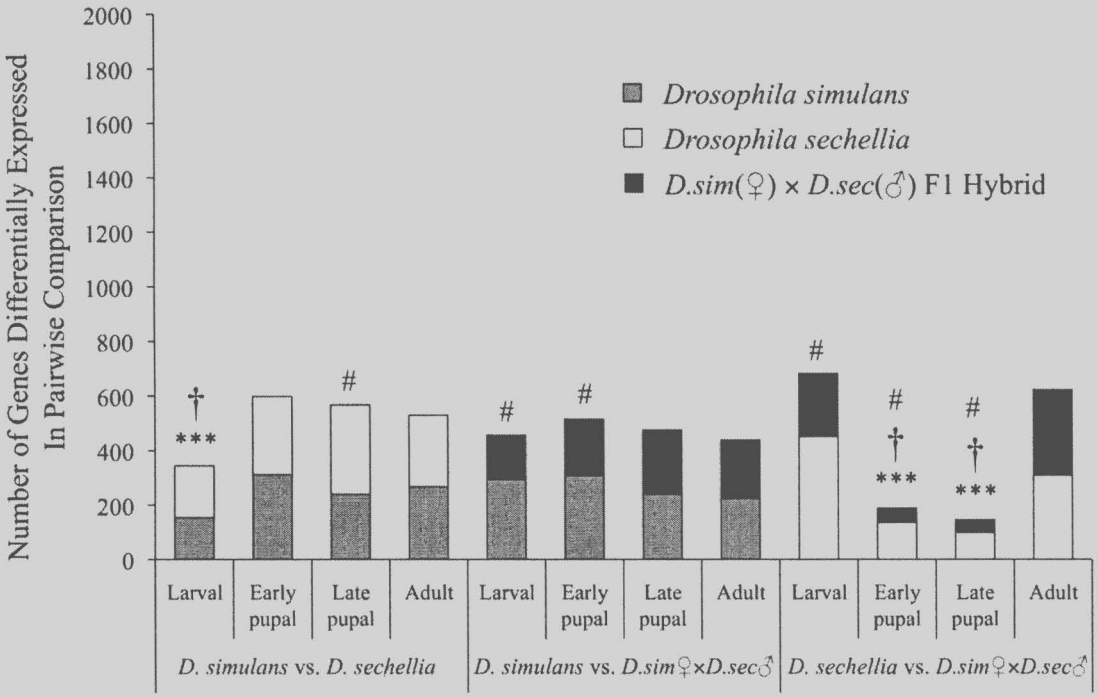


Figure 5.3: Number of genes significantly differentially expressed in pairwise comparisons between *D. simulans*, *D. sechellia*, and the *D. simulans* (♀) × *D. sechellia* (♂) F1 hybrids (*D.sim*♀×*D.sec*♂) at each of the four sampled stages (out of 2,052 genes). The comparison between each species is divided into two segments, each indicating the number of genes that are expressed at a higher level in the species of the corresponding shade: grey, *D. simulans*; white, *D. sechellia;* and black *D.sim*♀×*D.sec*♂ (see the legend in the upper-right hand corner of the figure). In the comparison between *D. simulans* and *D. sechellia* there are fewer genes differentially expressed at the 3rd instar larva stage than any other stage as indicated by the three asterisks (***) ($P < 0.001$). Similarly, in the comparison between *D. sechellia* and *D.sim*♀×*D.sec*♂ both the early and late pupal stages show significantly fewer genes that are differentially expressed as compared to the 3rd instar larval or adult stages again as indicated by the three asterisks ($P < 0.001$). All other between stage comparisons within the pairwise comparisons were not statistically significant. The daggers (†) indicate stages where the number of genes differentially expressed in the pairwise comparison between species is significantly lower than both other between species comparisons at the same stage ($P < 0.001$). Hash symbols (#) indicate comparisons where there is significant (χ^2 goodness of fit test, $P < 0.05$) asymmetry in the number of genes that are expressed at a higher level in one species vs. the other.

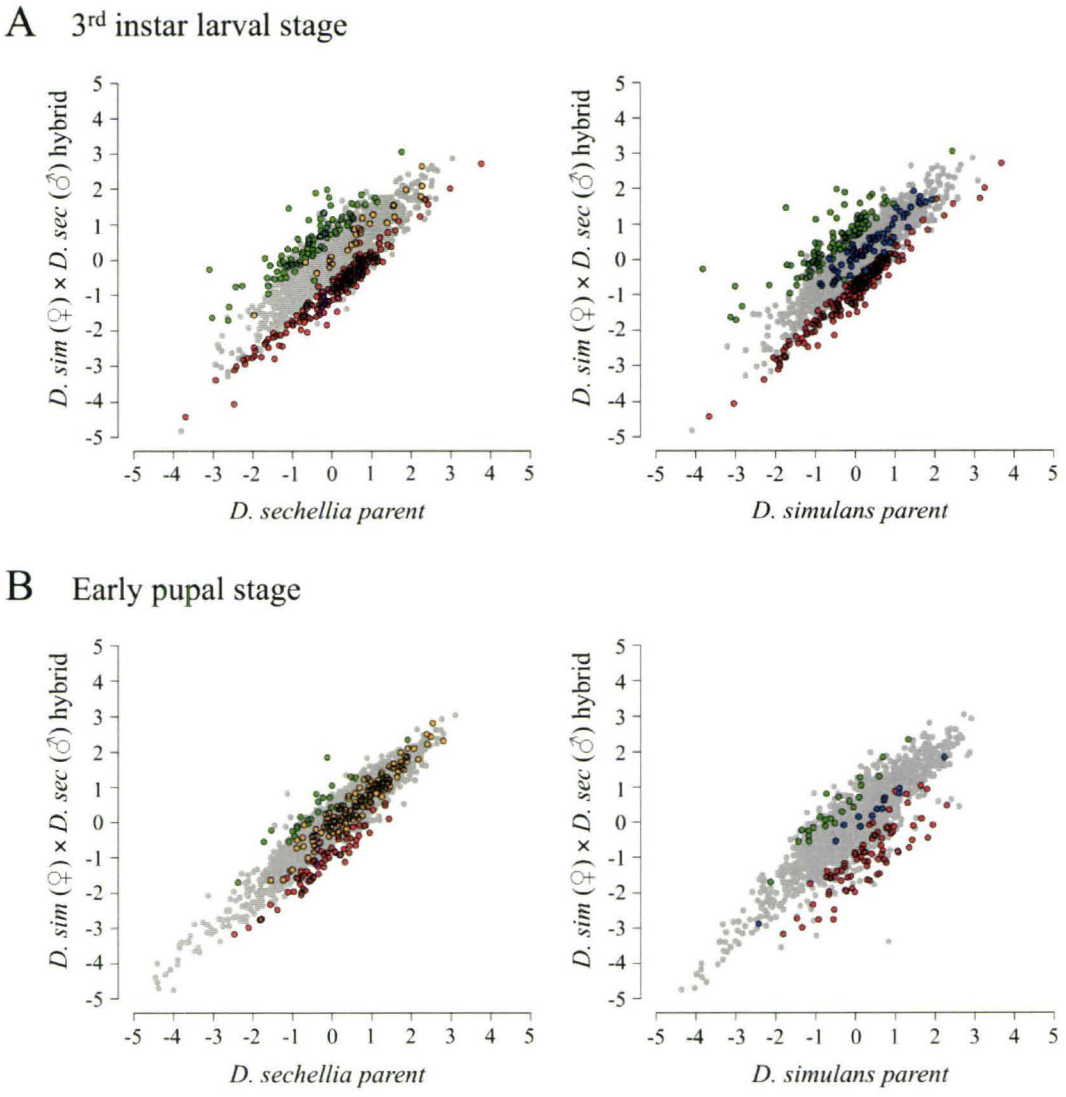


Figure 5.4: Scatterplots comparing the $\log_2$(sample/reference) expression values between each of the parental species (*D. sechellia*, left; *D. simulans*, right) vs. the *D. simulans* (♀) × *D. sechellia* (♂) F1 hybrids at the (**A**) 3rd instar larval and (**B**) early pupal stages. Colors indicate genes classified based on their expression pattern in the hybrid relative to the parents: green: genes overexpressed in the hybrid relative to both parents; red, genes underexpressed in the hybrid relative to both parents; orange, genes significantly differentially expressed between parental species and expressed at *D. sechellia* levels in the hybrid; blue, genes significantly differentially expressed between parental species and expressed at *D. simulans* levels in the hybrid; grey, all other genes. The number of genes represented in each category is shown in Table 5.1.

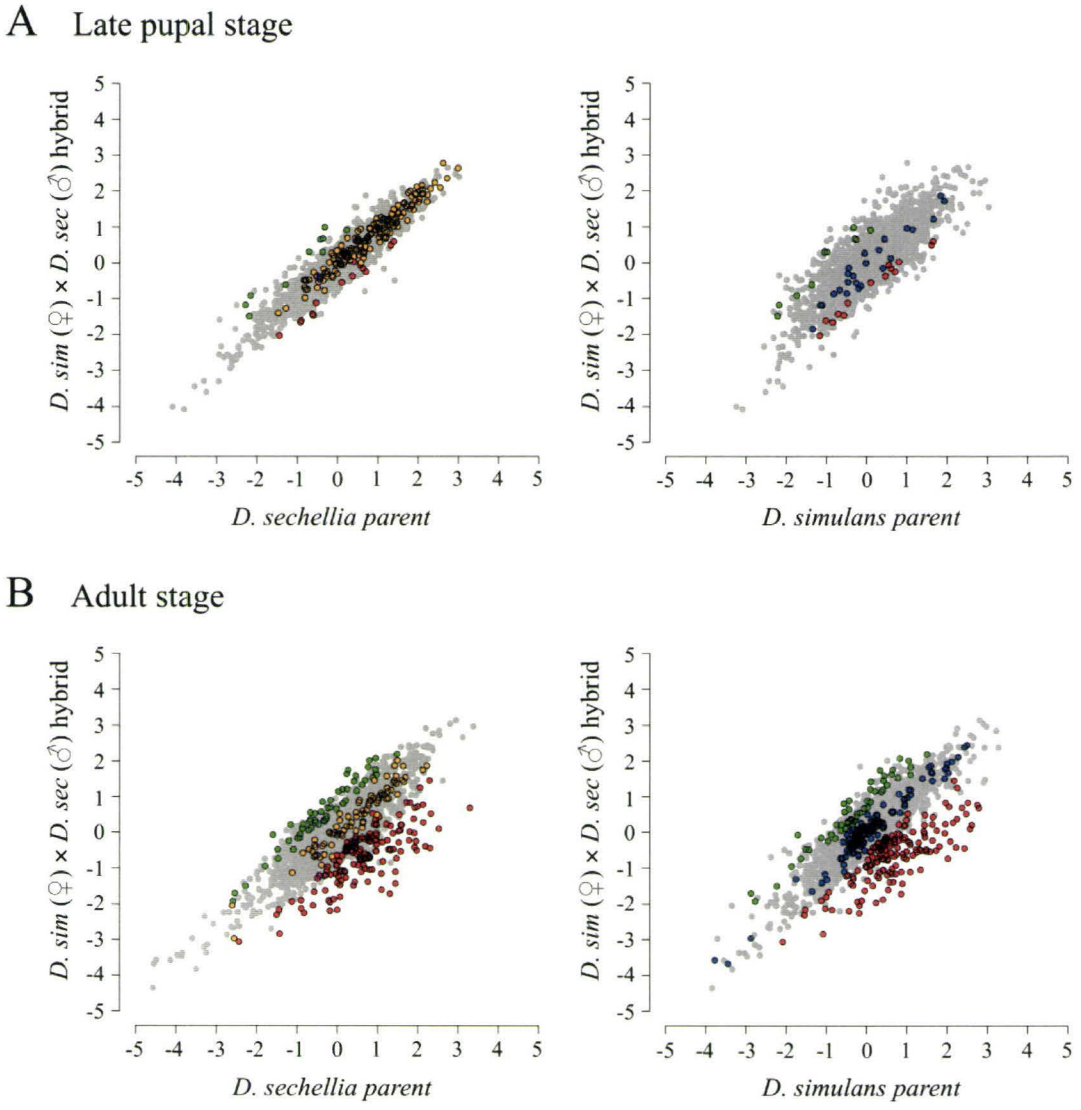


Figure 5.5: Scatterplots comparing the $\log_2$(sample/reference) expression values between each of the parental species (*D. sechellia*, left; *D. simulans*, right) vs. the *D. simulans* (♀) × *D. sechellia* (♂) F1 hybrids at the (**A**) late pupal and (**B**) adult stages. Colors indicate genes classified based on their expression pattern in the hybrid relative to the parents: green: genes overexpressed in the hybrid relative to both parents; red, genes underexpressed in the hybrid relative to both parents; orange, genes significantly differentially expressed between parental species and expressed at *D. sechellia* levels in the hybrid; blue, genes significantly differentially expressed between parental species and expressed at *D. simulans* levels in the hybrid; grey, all other genes. The number of genes represented in each category is shown in Table 5.1.

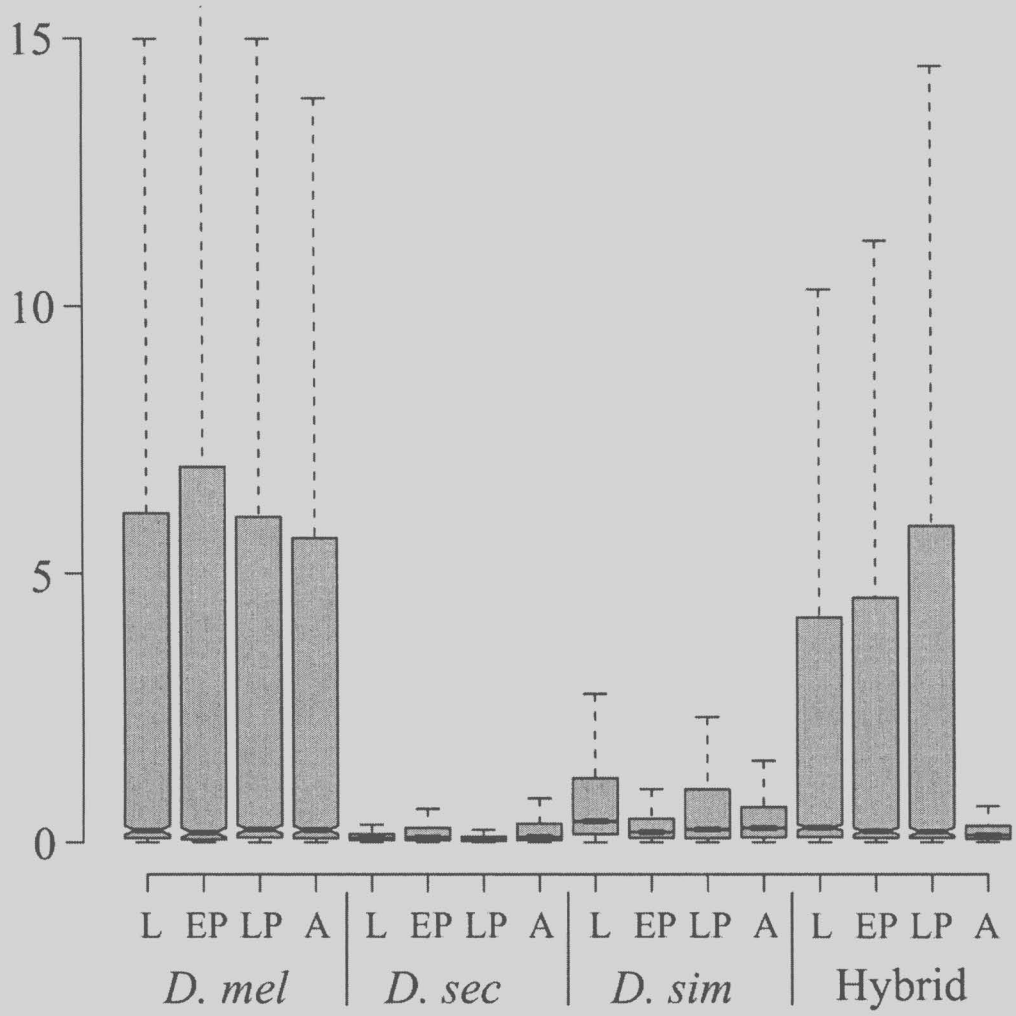
Table 5.1: Number of genes classified into categories based on their patterns of expression in *D. simulans* (♀) × *D. sechellia* (♂) F1 hybrids relative to the parental species. Patterns are as follows: Not differentially expressed, genes that are not significantly differentially expressed in any pairwise comparison among parental species or hybrids; Intermediate expression, genes expressed at a significantly intermediate level between parental expression levels; *D. simulans* dominance, genes significantly differentially expressed between parental species but for which the hybrids are not significantly different from *D. simulans* but are significantly different in expression level from *D. sechellia*; *D. sechellia* dominance, opposite of previous pattern; Underexpressed, genes that are significantly underexpressed in the hybrids relative to both parents; Overexpressed, genes that are significantly overexpressed in the hybrids relative to both parents; Other, genes whose expression patterns did not fit into any of the above classes. Stage abbreviations are as follows: Larval, 3rd instar larval; E. pupal, Early pupal; L. pupal, Late pupal.

	Stage			
Pattern	**Larval**	**E. pupal**	**L. pupal**	**Adult**
Not differentially expressed	1216	1455	1479	1203
Intermediate expression	0	9	24	8
D. simulans dominance	60	13	24	146
D. sechellia dominance	27	146	174	83
Underexpressed	206	76	14	149
Overexpressed	97	23	10	53
Other	446	330	327	410

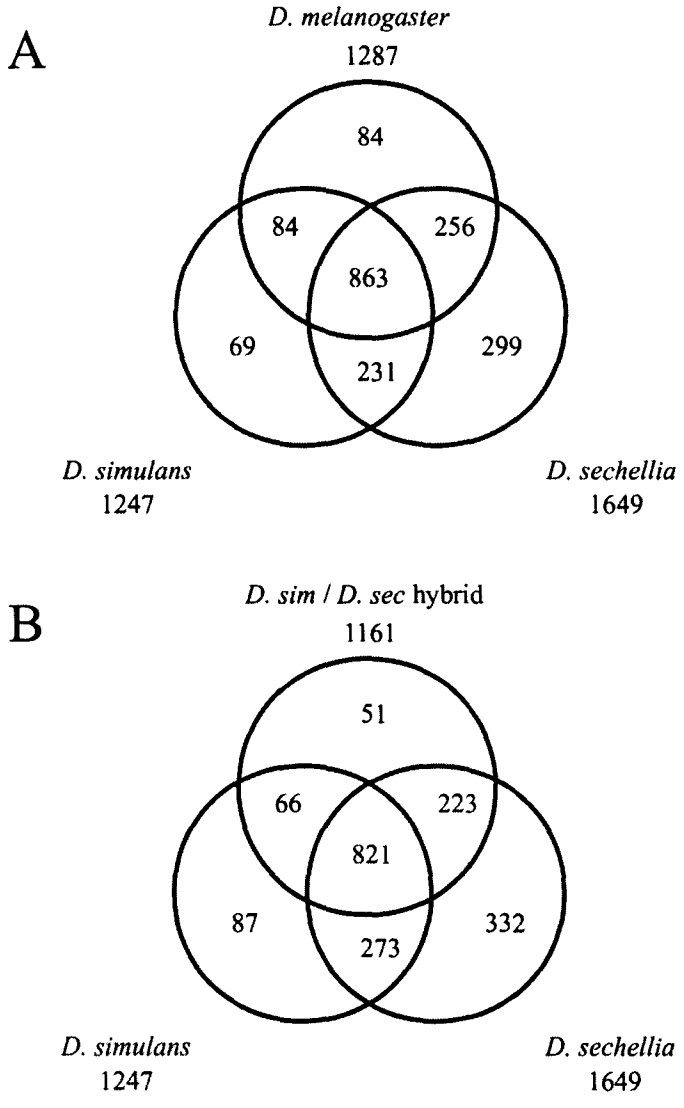
Table 5.2: Mean rates of divergence (d_N, d_S, and d_N/d_S) of genes that vary significantly in expression level among the 4 developmental stages studied as compared to those genes that are stably expressed. Permuted 95% confidence intervals are shown below the mean values. The columns labeled 'sig' indicate the p value of the significance of a pairwise permuted Kruskal-Wallis rank sum test of the difference between the distributions of genes that vary in expression level over development vs. those that do not vary for the divergence statistic to its immediate left; abbreviations are as follows: n.s., non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Species abbreviations are as follows: *D. mel*, *Drosophila melanogaster*; *D. sec*, *D. sechellia*; *D. sim*, *D. simulans*; Hybrid, *D. simulans* (♀) × *D. sechellia* (♂) F1 hybrids.

Species	**Vary**	**#**	d_N	**Sig.**	d_S	**Sig.**	d_N/d_S	**Sig.**
D. mel	No	492	0.1324	***	1.7759	***	0.0758	n.s.
			0.1210-0.1438		1.724-1.828		0.0693-0.0824	
	Yes	842	0.1151		1.6435		0.0710	
			0.1073-0.1230		1.604-1.682		0.0659-0.0760	
D. sec	No	249	0.1347	***	1.7096	n.s.	0.0838	**
			0.1198-0.1494		1.647-1.773		0.0735-0.0941	
	Yes	1085	0.1185		1.6884		0.0702	
			0.1112-0.1257		1.653-1.724		0.0660-0.0745	
D. sim	No	529	0.1385	***	1.7816	***	0.0813	n.s.
			0.1267-0.1503		1.733-1.830		0.0740-0.0885	
	Yes	805	0.1104		1.6337		0.0671	
			0.1029-0.1178		1.593-1.674		0.0627-0.0715	
Hybrid	No	585	0.1305	***	1.7521	**	0.0763	n.s.
			0.1204-0.1405		1.706-1.798		0.0701-0.0824	
	Yes	749	0.1145		1.6457		0.0699	
			0.1059-0.1232		1.603-1.687		0.0648-0.0751	

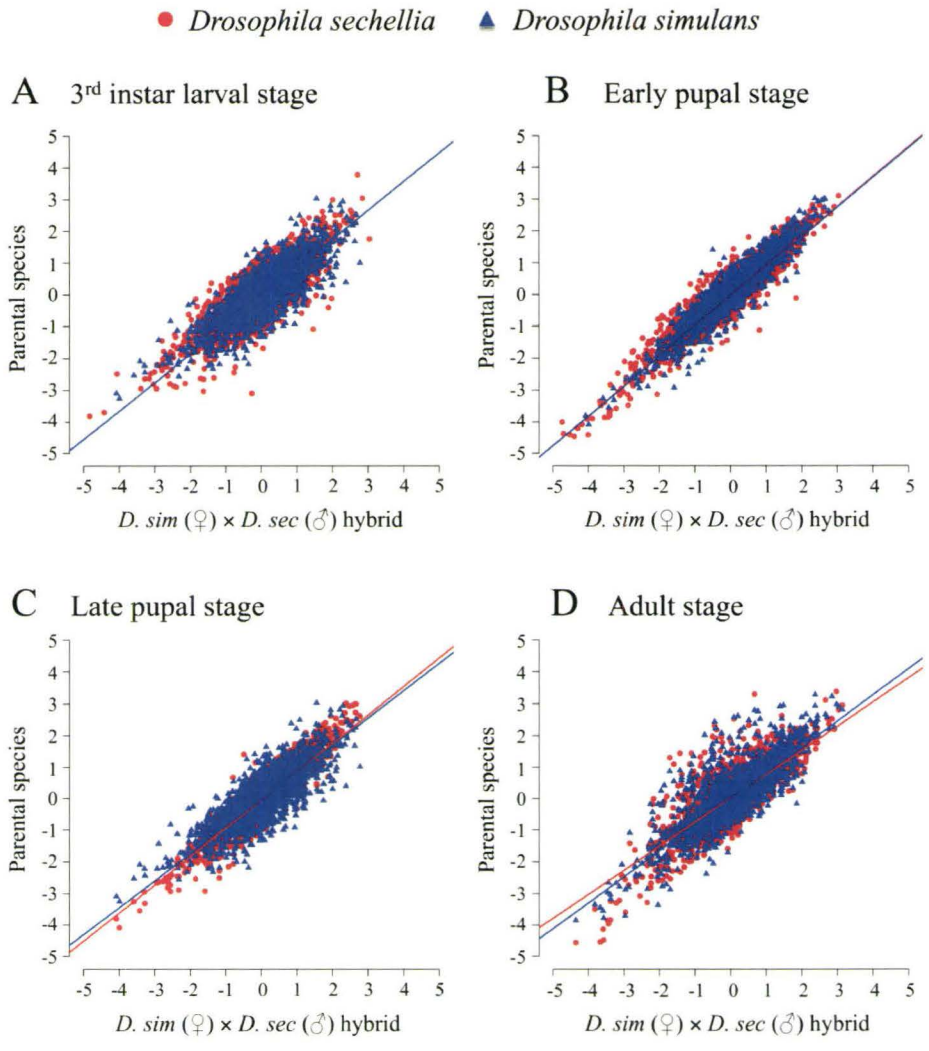
5.8 Supplementary Figures



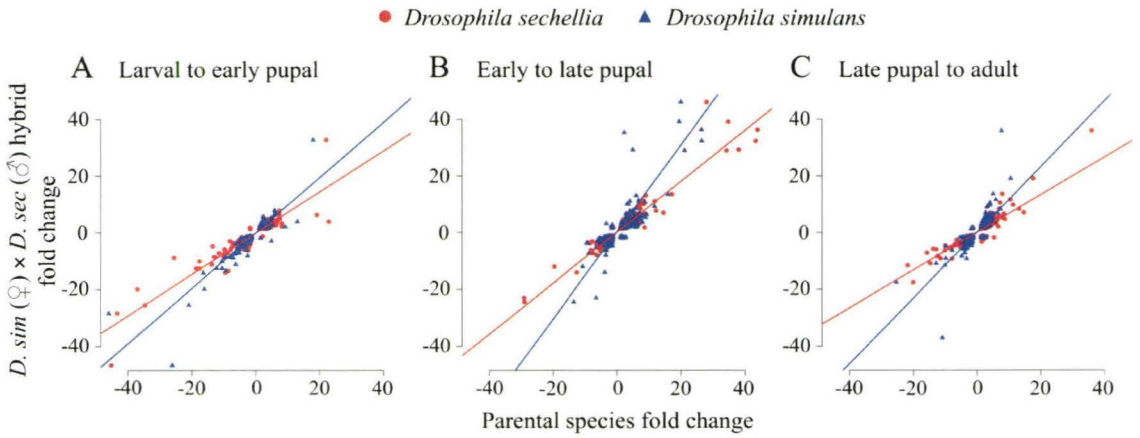
Supplementary Figure 5.1: Boxplots comparing the distribution of between microarray spot replicate variances for each stage (L, 3rd instar larval; EP, early pupal; LP, late pupal; A, adult) within the three pure species (*D mel*, *D. melanogaster*; *D. sec*, *D. sechellia*; *D. sim*, *D. simulans*) and the *D. simulans* (♀) × *D. sechellia* (♂) F1 hybrids (Hybrid). Note that the mean variance is lower in D. sechellia during the L, EP, and LP stages as compared to all other species/hybrids (permuted Kruskal-Wallis rank sum test, $P < 2.2 \times 10^{-16}$). Also, no stages in the hybrids showed the highest mean variance in comparisons with the three pure species, despite the hybrid replicate spots representing biological replicates.



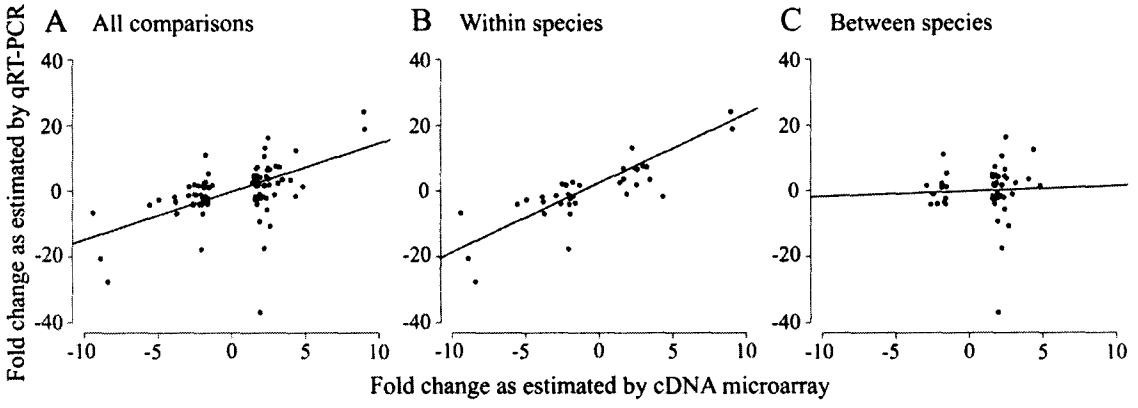
Supplementary Figure 5.2: Venn diagrams indicating the number of genes varying significantly in expression level over the 4 sampled developmental stages that are shared among (**A**) the three pure species and (**B**) the two parental species and the hybrids. The number of genes indicated in each segment of each the diagram is drawn from the 2,006 genes that were detectably expressed in all 3 species and the hybrid at all 4 sampled developmental stages. The numbers under each species' name indicates the total number of genes that vary significantly in expression level over the sampled developmental stages in that species/hybrid.



Supplementary Figure 5.3: Scatter plots comparing the levels of expression for all genes detectibly expressed in *D. simulans*, *D. sechellia*, and the *D. sim* × *D. sec* hybrid at the (**A**) 3rd instar larval, (**B**) early pupal, (**C**) late pupal, and (**D**) adult stages. Both axes indicate the $\log_2$(sample/reference) expression ratio. The comparison and linear regression between *D. sechellia* and the hybrid is shown in red (circles), while the comparison and linear regression between *D. simulans* and the hybrid is shown in blue (triangles). Only in the case of the adult stage is the hybrid more significantly correlated with one parent (*D. simulans*) as determined by ANCOVA ($F_{1,2050} = 33.724$, $P = 7.347 \times 10^{-9}$). Note that in stages where two regression lines are not visible, this is because both are superimposed upon one another.



Supplementary Figure 5.4: Scatter plots comparing the fold change in expression level between the hybrids and the two parental species for each of the three consecutive developmental transitions: (**A**) L to EP, (**B**) EP to LP, and (**C**) LP to A. The comparison and linear regression between *D. sechellia* and the hybrid is shown in red (circles), while the comparison and linear regression between *D. simulans* and the hybrid is shown in blue (circles). The slopes of the regression lines are all significantly different as determined by ANCOVA. The fold change in expression level between transitions in the hybrid is more significantly correlated to the *D. simulans* parent during the L to EP and LP to A transitions, while in the case of the EP to LP transition, the hybrid is more significantly correlated with *D. sechellia*.



Supplementary Figure 5.5: Scatter plots comparing the fold change in expression level (**A**) both within species between transitions and between species within stages, (**B**) using only within species data, and (**C**) using only between species data measured either by microarray or qRT-PCR. The linear regression, as shown by the solid line, is significant and positive ($P < 0.05$) in the case of the comparison of all data and within species data. However, both the line regression and the correlation become non-significant when only between species data is considered.

CHAPTER 6

GENERAL CONCLUSION

"[A] study of the effects of genes during development is as essential for an understanding of evolution as are the study of mutation and that of selection."

-Julian Huxley, *Evolution: The Modern Synthesis* (1942)

6.1 Concluding remarks

The study of development was largely neglected during the 'modern evolutionary synthesis' of population and Mendelian genetics with natural selection, and researchers in the field of the evolution of development have rightfully argued that no complete theory of evolution can ignore ontogeny (e.g., Carroll 2005a; 2008). Perusal of the current literature in the field of evolutionary biology will quickly reveal an abundance of articles suggesting that the evolution of gene regulation has played a disproportionate role (in comparison to traditional models of evolution by altering coding sequences) in determining the origins and evolution of some of the most intriguing phylogenetic patterns, such as the origin and maintenance of animal phyla, or those specific changes that explain 'what makes us human' (Davidson and Erwin 2006; King and Wilson 1975). While such speculation has certainly opened new avenues of research, notably the field of evo-devo, it has also engendered criticism from researchers concerned with the extreme position taken by some developmental biologists that traditional notions derived from the study of evolutionary biology, most notably population genetics, are incompatible with our current understanding of the evolution of development (Coyne 2006; Hoekstra and Coyne 2007). As stated in the introduction to this thesis, the overall goal of the material presented herein has been to study patterns of divergence between species, both in terms of nucleotide sequence, as well as gene regulation, with a special focus on development. These goals were undertaken in order to gain a better understanding of the population-level processes that lead to the interspecific differences in patterns of divergence in the context of organismal ontogeny (Johnson 2007). Three major results of our work warrant further discussion:

Firstly, our results suggest that both gene nucleotide sequences (see § 4) and expression levels (see § 5) follow the pattern of divergence over ontogeny predicted originally by Von Baer's (1828) classic observations, and reformulated into Raff's (1994;1996) 'developmental hourglass' hypothesis: certain stages of development (typically those occurring earlier in the ontogenic hierarchy) are generally more conserved between species than others. In the case of nucleotide sequence divergence, we

find evidence that two non-mutually exclusive processes may drive these patterns: developmental constraint acting on highly connected, developmentally important genes, and also increased opportunity for selection acting on divergence of those genes expressed during stages in which organisms are exposed to a greater variety of environmental variables – exemplified especially by the process of sexual selection (Figure 4.3) (Darwin 1859, 1882). Secondly, while we do find evidence for a correlation of sequence and expression divergence between species (see § 3 and 5), particularly in the case of divergence between adults, it nonetheless appears that these two levels of the overall progression from genotype to phenotype are subject to substantial uncoupling during ontogeny. Specifically, while we observed a general trend towards increased divergence of coding sequence of genes expressed at their highest levels in subsequent stages of ontogeny in *D. melanogaster* and *D. sechellia*, the mechanisms underlying gene regulation seem to be most conserved during an intermediate stage (pupation) as revealed in our analysis of the patterns of expression in interspecific hybrids. Finally, we find that the widespread misexpression of genes in interspecific hybrids relative to their parents appears to result from stage-specific, largely autonomous incompatibilities. This contradicts previous speculation suggesting that we are observing the end-result of a developmental cascade invoving a smaller number of misexpressed genes in ontogeny (e.g., Reiland and Noor 2002; Ortíz-Barrientos et al. 2007).

Such observations have important implications in the overall effort to synthesize evolution and development from the perspective of population genetics. Firstly, it is clear that understanding at what stage(s), and in what developmental context(s), genes are expressed is critical to generating an overall picture of the forces that underlie the patterns that we observe in our studies of comparative genomic data. Such a notion has been addressed by several recent papers in the context of spatial patterns of gene expression (i.e., broadly expressed genes appear to be more constrained in divergence as compared to genes with more restricted expression); and our data suggest that 'when' may be at least as important as 'where' when it comes to understanding the evolution of individual genes (Khaitovich et al. 2005; Haerty et al. 2007; Larracuente et al. 2008). Secondly, an understanding of causality relationships among various levels of the complex pathway between genotype and phenotype is crucial to opening that 'black-box' of population genetics, and ultimately allow more accurate modeling of how organisms evolve (Johnson and Porter 2001). Several recent studies have stressed the point that understanding evolution ultimately requires more biologically realistic models of how mutations at various levels in the overall biological hierarchy ultimately affect phenotypes (e.g., Lemos et al. 2005). Our data, combined with those of other studies, suggest that expression patterns have a significant role in constraining and shaping coding sequence divergence, but may themselves be subject to alternative selection pressures from those affecting exonic sequences – as exemplified by the observation that late pupal stage genes in *Drosophila* are often more diverged in terms of coding sequence, but more conserved in terms of underlying regulatory factors (see Chapters 4 and 5). These observations may

provide support from the study of closely related species to the hypothesis advanced by members of the evo-devo community that patterns of gene expression may enjoy more freedom in terms of their ability to diverge between species as a result of the modularity of *cis*-regulatory elements minimizing the deleterious effects of pleiotropy associated with changes in coding sequences (Carroll 2005a, 2008).

Attempts have been made to integrate both gene regulation and developmental principles into population genetics models (e.g., Rice 1998, 2000; Hansen and Wagner 2001; Wolf et al. 2001); however, the degree to which such models approximate biological reality is contingent upon the quantity and quality of the data available regarding the patterns and processes leading to the evolution of the genome in the context of ontogeny. So long as developmental biology focuses upon the elucidation of universal patterns observed across vast phylogenetic distances, it will remain difficult to obtain an adequate understanding of the population-level processes from which these patterns ultimately derive. Thus, studies such as those presented in this thesis, focusing on comparative genomics in the context of the development of closely related species, are sorely needed in order to bridge the gulf between evo-devo enthusiasts and the 'mainstream' of evolutionary biology.

Ultimately the antagonism between population geneticists and developmental biologists may be a proverbial dead-letter, maintained by aficionados of the latter camp who insist that their field will offer revolutionary insights that will undermine traditional Darwinian notions (e.g, Davidson and Erwin 2006). Natural selection acts on the phenotype whose manifestation is ultimately governed at some level by the complex rules orchestrating the process of ontogeny. While understanding *how* phenotypes evolve requires knowledge of development, understanding *why* phenotypes evolve requires knowledge of population genetics. Carroll (2005b) may have rightfully referred to the field of evo-devo as a 'revolution' in modern biology. Furthermore, his likening of the insights gleaned from developmental genetics to those of the modern synthesis are particularly apt, as the modern synthesis did not eliminate Mendelian genetics, nor mutation, nor natural selection, but rather combined them to their mutual enrichment. What we have learned from the study of population genetics cannot be supplanted by new details emerging from evo-devo, but rather it should enjoy a similar enrichment from the nascent study of the 'micro-evolution of development' (Johnson 2007).

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