Developmental and Genetic Mechanisms of Ovariole Number Evolution in Drosophila

Developmental and Genetic Mechanisms of Ovariole Number Evolution in *Drosophila*

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Abstract

The goal of the "Quantitative Trait Gene" (QTG) program is to identify genes and mutations that underlie natural phenotypic variation. My goal with this work was to contribute an additional model to the program: ovariole number evolution in *Drosophila*. In this thesis I describe the progress I have made towards identifying a specific genetic change that contributed to the divergence of ovariole number between two *Drosophila* lineages. I identify specific developmental mechanisms relevant to establishing ovariole number in different *Drosophila* lineages by detailing ovarian cell-type specific specification, proliferation, and differentiation. I test specific candidates of genetic regulators of these developmental mechanisms with mutational analysis in *D. melanogaster*. I show that independent evolution of ovariole number has resulted from changes in distinct developmental mechanisms, each of which may have a different underlying genetic basis in *Drosophila*. I use the interspecies comparison of *D. melanogaster* versus *D. sechellia* to test for functional differences in insulin/insulin-like growth factor (IIS) signaling between the two species. I show that IIS activity levels and sensitivity have diverged between species, leading to both species-specific ovariole number and species-specific nutritional plasticity in ovariole number. Moreover, plastic range of ovariole number correlates with ecological niche, suggesting that the degree of

nutritional plasticity may be an adaptive trait. My work and quantitative genetic analyses strongly support the hypothesis that evolution of the *Drosophila insulin-like receptor* (*InR*) gene, specifically, is at least partially responsible for the divergence in ovariole number and nutritional plasticity of ovariole number between *D. melanogaster* and *D. sechellia*. I detail ongoing experiments to test this hypothesis explicitly via cross-species transgenesis.

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Chapter One

Introduction

Introduction

The Theory of Genetic Evolution

The integration of Mendelian genetics into the study of organismal evolution, a field that was, up to that point, dominated by studies in zoology, paleontology, systematics and biogeography, initiated a new theory of *genetic* evolution. The confluence of ideas would come to be known as the Modern Synthesis of evolutionary biology, underscoring the importance of this conceptual transition. The substrate for generating biodiversity, by natural selection as proposed by Darwin and Wallace, or by genetic drift or migration, is heritable variation within genes. The objective of the genetic theory of evolution is to uncover and explain the rules, patterns, and processes that describe how genes generate biodiversity.

As the definition of gene form and function grows more complete, and ever more complex, so, too, does the theory of genetic evolution. The first half of the twentieth century saw great progress in the theory through the Modern Synthesis. Population genetics, a result of the mathematical formalization of Mendel's laws, predicted the frequency of genetic variants (alleles) in populations as a result of their impact on organismal fitness. Dobzhansky (Dobzhansky, 1937), Simpson (Simpson, 1944), and others showed that the predictions from population genetic models accurately reflected the biology of extant natural populations and the paleontological record (reviewed in Huxley, 1942).

However, at the time when the Modern Synthesis was being developed, relatively little about gene structure and function was known. In the following decades, fundamental understanding of the gene would transform our understanding of the

constraints on genetic evolution. The solution of the structure of DNA in 1953 provided the critical clue to how DNA is copied and faithfully inherited. In 1961 Jacob and Monod published their groundbreaking work on the *lac* operon (Jacob and Monod, 1961), uncovering important principles of gene expression regulation by *trans*-acting factors. This knowledge would highlight the occurrence of epistasis, and challenge a critical assumption of population genetic theory of independent gene function. Around the same time, elucidation of the genetic code would introduce the ideas of synonymous versus non-synonymous sites in codons, implying constraint on the evolution of individual nucleotide bases.

In the past few decades, molecular developmental biology has been absorbed into evolutionary theory, spawning the field of "evo-devo" (reviewed in Abouheif, 2008; Carroll, 2008; Mallarino and Abzhanov, 2012). Molecular developmental genetics, which seeks to understand how genes give rise to form through growth, differentiation and morphogenesis, the so-called "genotype to phenotype" problem, has refined our understanding of individual and collective gene function. Seminal findings that spurred the field were experiments that elucidated the "Hox code" and its conservation across metazoans (reviewed in Carroll et al., 2005). This work strongly supported the hypothesis that the diversity in animal body plans is largely the result of differential deployment of a common genetic toolkit versus differentiation of the toolkit in different lineages. Synthesis of decades of subsequent research has led to many new models and hypotheses about how genetic evolution may proceed, for example modularity or mesoevolution (reviewed in Abouheif, 2008; E. C. Raff and R. A. Raff, 2000).

A more complete theory of genetic evolution is best pursued through studies that span the range of biological complexity, from molecules and cells to populations and species. Revolutionary technological advances of the past two decades in microscopy, computing, and genomic sequencing capabilities give us unprecedented access to the full breadth of this range.

A New Model within the "Quantitative Trait Gene" Program

It is generally agreed that general principles of the theory of genetic evolution will be revealed only by the accumulation of empirical examples of the molecular basis of phenotypic evolution (Conte et al., 2012; Hoekstra and Coyne, 2007; Stern and Orgogozo, 2009). Indeed, the "Quantitative Trait Gene" (QTG) program, which has as its objective to identify the genes and mutations that cause observable phenotypic variation at various taxonomic scales in eukaryotic organisms, including plants, animals, and yeast, is in full swing (Martin and Orgogozo, 2013; Stern and Orgogozo, 2009). In a recent comprehensive survey, Martin and Orgogozo (2013) catalogued over 1000 alleles that have been demonstrated to underlie the evolution of phenotypic differences, collectively called the "Loci of Repeated Evolution" (Martin and Orgogozo, 2013). Excellent overviews and thorough discussions of individual examples appear elsewhere (Hoekstra and Coyne, 2007; Martin and Orgogozo, 2013; Nadeau and Jiggins, 2010). This thesis takes as its modest goal the addition of a new trait to the QTG program: ovariole number in *Drosophila*. Through the work presented in this thesis, I demonstrate that ovariole number in *Drosophila* is a useful model within the QTG program. I suggest that the

ovariole number model has the potential to contribute important insight into the theory of genetic evolution.

I continue this introductory chapter by motivating the choice of *Drosophila* ovariole number as a QTG program trait. I begin with a brief introduction to insect ovary form and function. Next, I summarize what is known about ovariole number diversity and describe the preliminary work done to identify genetic controllers of this divergence. I then introduce my approach taken here to extend the evolutionary genetic analysis of ovariole number via a closer study of ovariole number development. I summarize the knowledge contemporary to the inception of this work on ovariole number development and of the genetic regulation thereof. I conclude with the specific outstanding questions and problems that this thesis addresses.

Ovariole structure and function

The unusual structure of the insect ovary was first documented and illustrated by the famous insect biologist Morio Malphigi in 1669 during his studies of the silkmoth *Bombyx mori* (Büning, 1994). Striking in his illustration are long strings of pearls that connect at what eventually leads to the female external genitalia (Figure 1.1). Each pearl is an individual developing oocyte and each string is an ovariole. All insect ovaries are subdivided in such a way into independent functional units. Ovarioles are the site of oogenesis in females.

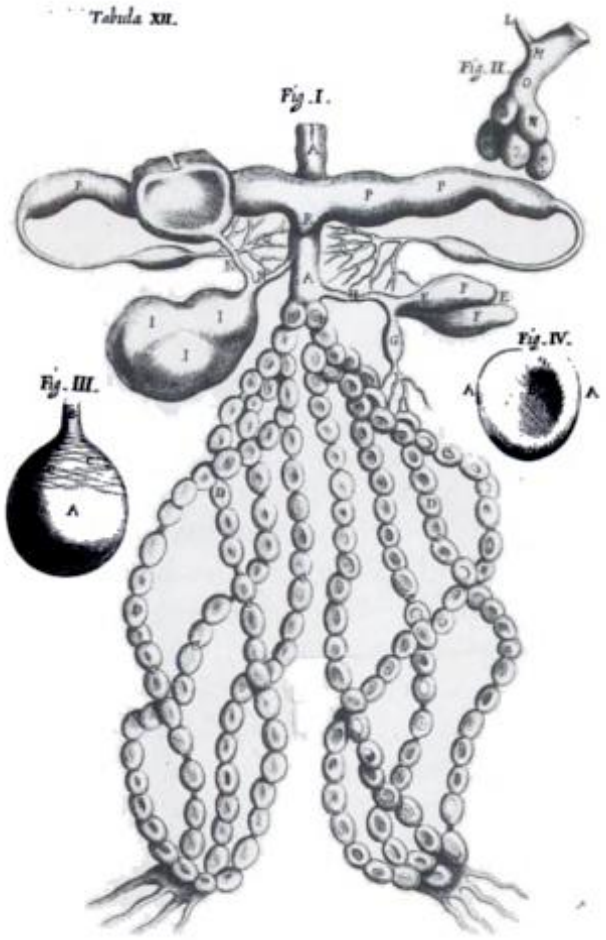

Figure 1.1: Illustration of the female reproductive system from *Bombyx mori* (silkmoth) by Morio Malphigi (1669). As described in the text, the "strings" are individual ovarioles, and the individual "pearls" are chorionated, mature eggs that are post vitellogenesis (yolk deposition). The left and right ovaries, each consisting of four ovarioles, converge at the common oviduct (depicted at the top of the page). This structure, which is typical for *Bombyx mori*, differs from that in *Drosophila*, in which individual ovarioles contain oocytes spanning the range of maturity, from germline stem cells to fully mature oocytes (Figure 1.2). Illustration from (Büning, 1994).

Ovariole structure is similar across insects. At the anterior tip is an organized group of somatic cells called the terminal filament (TF). Adjacent to the TF is the germarium, the place where oogenesis is initiated. Specific somatic cells within the germarium will constitute the germline stem cell (GSC) niche and direct proliferation and maintenance of the GSCs. Ovariole structure is broadly categorized in one of two ways, depending on the fate of oogonia. The putative ancestral type are panoistic ovarioles in which all oogonia either self-renew or eventually differentiate into a mature oocyte. Meroistic ovarioles, the category containing *Drosophila*, are ones in which germline cysts develop. Within cysts, one germline descendant is designated as the eventual oocyte and the remaining descendants acquire a supportive role and generate mRNAs and proteins for the single ooblast.[1] Despite these differences, insect ovary diversity is most apparent in the *number* of ovarioles that comprise a single ovary. This diversity is interesting given the direct relationship between ovary structure (ovariole number) and reproduction.

Ovariole number is related to female fecundity and reproductive fitness

Ovariole number is a strong determinant of female reproductive capacity (i.e. fecundity; I will use these terms interchangeably). Ovarioles function independently of one another and each produce an average of two eggs per day in periods of maximal egg production (Cohet and David, 1978). Maximum egg production rate, i.e. number of eggs produced per ovariole per day, is independent of the number of ovarioles a female has (Extavour, 2000). This leads to the expectation that egg production is directly

[1] A more thorough discussion of ovariole structure and oocyte development can be found in Appendix A.

proportional to ovariole number. Several reports confirm that ovariole number is broadly correlated to lifetime egg production, although the correlation is not absolute. Ovariole number variation is positively correlated with fecundity within populations of *D. melanogaster* (David 1970), between populations of *D. melanogaster* (Boulétreau-Merle et al., 1982; Klepsatel et al., 2013), and between closely related Drosophilids (R'kha et al., 1997). Klepsatel et al. (Klepsatel et al., 2013) recently conducted a longitudinal study of hundreds of individual female *D. melanogaster* flies, documenting ovariole number and different life history characteristics, including fecundity, hatchability and lifespan. These authors found that ovariole number is strongly correlated to peak fecundity in females, and is also correlated to lifetime fecundity.

Female fecundity is a critical determinant of fitness, and thus ovariole number directly impacts organismal fitness. The relationship, however, is complex. In experiments focused on ovariole number variation within a single population of *D. melanogaster*, Wayne et al. (1998) suggested that ovariole number is under stabilizing selection. The same authors found that in a separate, although less sensitive, assay a correlation between ovariole number and fitness was not observed (Wayne et al., 1997).[2] Nonetheless, when different lineages are compared, results consistently show a positive correlation between mean ovariole number for and relative fitness of a lineage (Klepsatel

[2] In Wayne and Mackay (1998), fitness was measured specifically for females in a single generation competitive test. Females with the genotype of interest, in this case from individual mutation accumulation lines, were allowed to lay eggs alongside marked (yellow) control females. The frequency of wild type versus marked progeny was scored. In Wayne et al. (1997), fitness was measured for males and females separately using Sved's multigenerational competition technique (Sved, 1971). This test, and multigenerational tests in general, are potentially confounded by issues of development time and longevity (Haymer and Hartl, 1983).

et al., 2013; R'kha et al., 1997). This correlation holds for other insects as well (Grenier and Nardon, 1994).

Ovariole number diversity is genetically and environmentally generated

Ovariole number has been studied for decades, and thus a substantial amount is known about ovariole number variation in wild and laboratory-maintained insects. Ovariole number is a straightforward morphology to identify, dissect, and measure with high accuracy using relatively simple tools (i.e. light microscopy). Ovariole number shows striking divergence, spanning over three orders of magnitude among insects (Büning, 1994; Hodin, 2009). The oil beetle *Meloe* has been reported to have an ovariole number topping 1000 per ovary (Hodin, 2009). Queen honeybees have a few hundred ovarioles per ovary (Linksvayer et al., 2009). Variation in ovariole number among insect orders differs substantially. For example, ovariole number within the Lepidoptera is fixed at four ovarioles per ovary (Hodin, 2009), whereas ovariole number within the Diptera, and even within the single genus *Drosophila*, spans two orders of magnitude (Hodin, 2009). Intraspecies variation also differs. Natural variation in ovariole number is significantly higher in *D. melanogaster* than in *D. simulans* (Capy et al., 1994; Gibert et al., 2004) and *D. sechellia* (Green and Extavour, 2014). Patterns of ovariole number evolution are not obvious when looking across the phylogeny of insects or within more specific clades, for example the Drosophila subgenus Sophophora (Figure 2.2). This is likely because, as discussed next, ovariole number is not only genetically determined, but also environmentally controlled.

A critical contributor to ovariole number variation is its phenotypic plasticity. As discussed below, larval stages are the critical period during which ovariole number is determined. As such, larval growth conditions strongly influence ovariole number, particularly nutrition (Bennettova and Fraenkel, 1981; Bergland et al., 2008; Hodin and Riddiford, 2000; Sarikaya et al., 2012; Tu and Tatar, 2003), temperature (Hodin and Riddiford, 2000; Sarikaya et al., 2012), and crowding (personal observation; (Capy et al., 1993)).

Ovariole number is correlated with ecology. Ovariole number shows clinal variation with respect to latitude (Capy et al., 1994; 1993; Gibert et al., 2004)and altitude (Collinge et al., 2006; Wayne et al., 2005) on different continents, strongly suggesting climatic adaptation. Temperate populations of the cosmopolitan *Drosophila* species *D. melanogaster* and *D. sechellia* have a larger ovariole number compared to tropical populations (Boulétreau-Merle et al., 1982; Capy et al., 1993; Klepsatel et al., 2013). Ovariole number is also correlated with host diet and has implications for the mechanism and evolution of reproductive strategy (Atkinson, 1979; Kambysellis et al., 1995). Flies that subsist on nutritionally abundant sources, or a variety of sources, tend to have increased ovariole numbers; in contrast, flies that are restricted to a particular niche, either obligately or facultatively, or a nutritionally deficient niche, tend to have reduced ovariole numbers (Green and Extavour, 2014; Kambysellis and Heed, 1971).

Preliminary genetic analyses have been conducted for ovariole number variation. Ovariole number is a quantitative trait. Coyne et al. (Coyne et al., 1991) and Wayne et al. (Wayne et al., 2001) showed that ovariole number is a polygenic trait controlled by "relatively few" loci restricted to the autosomes. With finer resolution, additional studies

confirmed the relative importance of the autosomes compared to the X chromosome as controllers to ovariole number variation (Bergland et al., 2008; Orgogozo, 2006). However, the loci identified in these studies were not fully concordant. These differences could be artifacts of the particular mapping technique used (Matute et al., 2009; Rebeiz et al., 2009). On the other hand, these differences may also reflect real biological differences resulting from different experimental setups (e.g. inter- versus intra-species mapping, gene-by-environment interaction under different nutritional regimes, etc.).

Development as a complementary approach to uncovering genetics of variation

At the time this work commenced, the lower bound for the number of protein-coding genes controlling intra- and inter-species ovariole number variation was approximately 6850 and 34, respectively (Orgogozo, 2006; Wayne and McIntyre, 2002). In these studies, classical F_2 backcross mapping approaches were complemented with microarray analysis or selective phenotyping to refine the coarse resolution of initial QTL experiments. Finer scale mapping by increasing the phenotyping effort quickly grows intractable, however, due to the nature of the phenotype. Although ovariole number is an easy phenotype to score, it is currently requires a relatively labor-intensive dissection and manual counting.[3]

In the work presented herein, I describe a complementary approach to identifying loci that contribute to ovariole number variation in *Drosophila*. I complement

[3] Some groups use a procedure in which they dissect ovaries, DAPI-stain and squash-mount them, and count ovariole number from collected fluorescence micrographs. Although this method allows a record of ovariole number to be maintained for a period of time, it does not preclude manual counting, which is the most labor-intensive step. Automated methods to accurately count ovariole number would make larger scale mapping studies more feasible.

quantitative genetics analyses with an understanding of the developmental mechanisms controlling ovariole number determination. With this information I choose candidate genes responsible for ovariole number variation for further functional analysis. An introduction to the developmental genetics of ovary development in *Drosophila* follows.

Ovary development in Drosophila

Ovary development in *Drosophila* was initially described by Julius Kerkis in 1931 (Kerkis, 1931). Kerkis recognized that the gonads are similar to imaginal tissues in *Drosophila* in that they grow throughout larval and pupal life. Growth rate differs substantially between the testis and ovary, evident as early as the beginning of larval life. Kerkis recognized differentiation of ovarian tissues into "egg-strings" (i.e. ovarioles) only within the first hours following pupariation.

Robert King (King et al., 1968); reviewed in (King, 1970) significantly refined the description of ovarian development, with particular focus on late larval and pupal stages. Like Kerkis, King observed delineated ovarioles by two hours after puparium formation. Moreover, King recognized and described distinct cell types and morphogenetic processes within the ovary. At the anterior tip of each ovariole is a stack of 9-10 disc-shaped cells called the "terminal filament" (TF) (Figure 1.2). A basal membrane, the tunica propia, is secreted from somatic cells in the anterior of the ovary that migrate posteriorly, initially using individual TFs as guides. Within the tunica propria is ensheathed a single TF, a small pool of primordial germ cells, and a pool of posterior somatic cells that eventually become interfollicular stalk and basal stalk cells. Based on these observations, King hypothesized that the number of TFs at entry to

pupariation determined the number of ovarioles in the adult. Hodin and Riddiford (Hodin and Riddiford, 1998) showed this to be the case. Furthermore, King suggested that the relevant time period for understanding TF number determination must be in embryonic and/or larval stages.

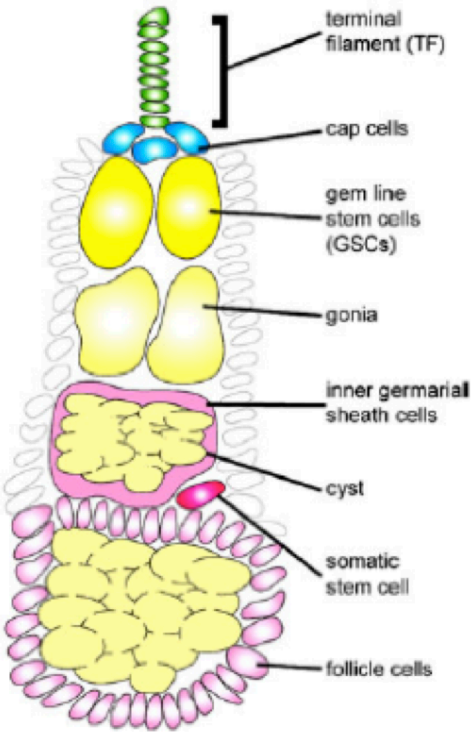

Figure 1.2: Anatomy of an adult female ovariole in Drosophilids. Germarium of an adult female ovary. Oogenesis begins at the anterior tip of the gonad (green terminal filament cells, blue cap cells, dark yellow germ-line stem cells [GSCs], light yellow gonia and differentiating gametogenic cells [cysts], dark pink follicle [somatic] stem cells, light pink follicle cells). Anterior is up. This figure is adapted from Green et al., 2011.

Embryonic gonadogenesis in *Drosophila* is relatively well understood. Somatic gonad precursor cells (SGPs) are specified within the mesoderm of parasegments 10-12 (Boyle and DiNardo, 1995). Homeotic genes *abdominal A* and *Abdominal B* cooperatively specify posterior SGPs, while *abdA* specifies anterior SGPs (Boyle and DiNardo, 1995). Anterior and posterior SGPs assume distinct fates in the larval ovary. *clift*/*eyes absent* maintains SGP fate after specification (Boyle et al., 1997). *even-skipped, engrailed*, *wingless*, *tinman*, and *bagpipe* expression further distinguish SGPs from other mesodermal subpopulations (Boyle et al., 1997; Riechmann et al., 1998). SGPs and pole cells eventually coalesce in parasegment 10 to form the embryonic gonad.

Dorothea Godt and others have contributed important details to the description of larval ovary and TF morphogenesis. Sahut-Barnola et al. (Sahut-Barnola et al., 1996) showed in BrdU pulse-chase experiments that TF precursor cells (TFPCs) proliferate throughout larval life, but upon joining stacks no longer divide. This led to the idea that a pool of TFPCs exists that are sorted into TF stacks, hence determining ovariole number. Sarikaya and colleagues (Sarikaya et al., 2012) determined that TFPC number, as opposed to TFPC size or TF morphogenesis mechanisms, is the most relevant genetically controlled parameter that determines TF number in *D. melanogaster*.

Genetic regulation of larval ovary development

A small number of genetic regulators of TF morphogenesis have been identified. Godt and Laski (Godt and Laski, 1995) identified *bric-á-brac* (*bab*) as a genetic regulator of ovary morphogenesis. The *Bab* locus contains two paralogous genes, *Bab1* and *Bab2*, both of which encode transcription factors. Both proteins share a BTB/POZ (Bric-á-brac

Tramtrack Broad complex/Pox viruses and Zinc-fingers) protein-protein interaction domain and Pipsqueak DNA-binding domain (Couderc et al., 2002; Lours, 2003). However, *Bab1* and *Bab2* have independent functions in the ovary. *Bab1* is expressed exclusively in TFPCs and TFCs. *Bab1* normally functions to reduce TFPC proliferation, as its loss of function leads to significant increase in TFC proliferation and TF number (Bartoletti et al., 2012). *Bab2* is expressed in all somatic cells of the larval ovary beginning in the earliest larval stages. *Bab2* affects somatic cell differentiation and morphogenesis prior to TF stacking, and is required for TFC differentiation and morphogenesis (Couderc et al., 2002). Assays utilizing loss of function mutations of both *bab1* and *Bab2* demonstrated that at least one or both proteins are required for TFC differentiation (Godt and Laski, 1995) and TF stacking (Bartoletti et al., 2012). Additionally, the segment polarity genes *engrailed* and *hedgehog* are expressed in TFPCs and TFCs, and controls the stacking ability of TFCs (Besse et al., 2005; Bolívar et al., 2006).

Hormone/neuroendocrine signaling also controls TF morphogenesis. Hodin and Riddiford identified the ecdysone nuclear hormone signaling pathway as a critical regulator of TF development and TFPC number determination (Hodin and Riddiford, 1998). Ecdysone signaling has both a non-autonomous effect, in which changes in the timing of metamorphosis alters TF stacking dynamics; and an autonomous effect, in which *ultraspiracle* is specifically required within TF cells for proper differentiation and subsequent alignment within stacks (Gancz et al., 2011; Hodin and Riddiford, 1998). Finally, it has recently been demonstrated that insulin and target of rapamycin (TOR)

signaling also control somatic cell proliferation, thus controlling TF number (Gancz and Gilboa, 2013; Green and Extavour, 2014; 2012).

Summary

My ultimate goal was to identify a specific genetic change that contributed to morphological evolution between two *Drosophila* lineages. In this work I do *not* demonstrate a particular causative mutation of evolutionary genetic change of ovariole number in *Drosophila*. Nevertheless, I do advance ovariole number determination in *Drosophila* as a trait within the QTG program through addressing the following questions:

1. *What developmental mechanisms are relevant to establishing TFPC pool number in different lineages?* I follow ovarian cell-type specific specification, proliferation and differentiation in different *Drosophila* lineages. (Chapter 2)
2. *What genetic mechanisms control these specific developmental mechanisms?* I perform mutational analysis in *D. melanogaster* on specific candidate genes based on data from previous quantitative genetic analyses and my ovary development studies. (Chapter 2)
3. *Do the genetic mechanisms controlling development function differently between lineages?* I use the interspecies comparison of *D. melanogaster* versus *D. sechellia* to test for functional differences in insulin/insulin-like growth factor (IIS) signaling between the two species. I also describe additional divergent phenotypes that arise due to differences in IIS activity (Chapter 3)

All of these experiments have led to a specific hypothesis for a locus of genetic change that has contributed to ovariole number divergence between *D. melanogaster* and *D. sechellia*. I detail experiments to test this hypothesis in Chapter 4.

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Chapter Two

Convergent Evolution of a Reproductive Trait Through Distinct Developmental Mechanisms in *Drosophila*

Abstract

Convergent morphologies often arise due to similar selective pressures in independent lineages. It is poorly understood whether the same or different developmental genetic mechanisms underlie such convergence. Here we show that independent evolution of a reproductive trait, ovariole number, has resulted from changes in distinct developmental mechanisms, each of which may have a different underlying genetic basis in *Drosophila*. Ovariole number in *Drosophila* is species-specific, highly variable, and largely under genetic control. Convergent changes in *Drosophila* ovariole number have evolved independently within and between species. We previously showed that the number of a specific ovarian cell type, terminal filament (TF) cells, determines ovariole number. Here we examine TF cell development in different *Drosophila* lineages that independently evolved a significantly lower ovariole number than the *D. melanogaster Oregon R* strain. We show that in these *Drosophila* lineages, reduction in ovariole number occurs primarily through variations in one of two different developmental mechanisms: 1) reduced number of somatic gonad precursors (SGP cells) specified during embryogenesis; or 2) alterations of somatic gonad cell morphogenesis and differentiation in larval life. Mutations in the *D. melanogaster* Insulin Receptor (*InR*) alter SGP cell number but not ovarian morphogenesis, while targeted loss of function of *bric-à-brac 2* (*bab2*) affects morphogenesis without changing SGP cell number. Thus, evolution can produce similar ovariole numbers through distinct developmental mechanisms, likely controlled by different genetic mechanisms.

Introduction

Convergent morphologies can evolve independently in different lineages, often as a result of similar selective pressures or functional requirements. An outstanding question in evolutionary and developmental biology is whether similar traits evolve convergently through changes in the same or different developmental and genetic processes. Changes in different processes suggest that natural selection may be the major force controlling form; changes in the same processes may suggest that development of the phenotype constrains how it can be modified over evolutionary time (Losos, 2011; Sanger et al., 2012). In recent years, several examples of convergent evolution at the molecular, cellular and morphological levels have been examined (Aminetzach et al., 2009; Moczek et al., 2006; Protas et al., 2006; Sucena et al., 2003; Tanaka et al., 2009; Wittkopp et al., 2003). In some of these cases, similar morphologies have evolved independently via changes in the same genes or genetic pathways (Chan et al., 2010; Protas et al., 2006; Prud'homme et al., 2006; Sucena et al., 2003; Zhang et al., 2012). However, in other cases convergent evolution of similar traits arises through different developmental or genetic mechanisms (; Shapiro et al., 2009; Steiner et al., 2009; Tanaka et al., 2009; Wittkopp et al., 2003; Zwaan et al., 2000).

In many of the cases where the genetic basis is well understood, the convergent trait hinges on the terminal differentiation of a single cell type, such as pigmentation (; Prud'homme et al., 2006; Steiner et al., 2009; Wittkopp et al., 2003) or sensory bristles (). However, there are few well-studied examples in which the convergent trait involves a multicellular structure composed of many distinct cell types (; Tanaka et al., 2009; Zwaan et al., 2000). Moreover, while many external anatomical traits have been studied in this

context, the evolution of internal reproductive morphologies that directly affect fecundity are less well understood. As a step towards elucidating the genetic mechanisms underlying the evolution of reproductive morphologies, here we examine changes in development that lead to major differences in ovariole number, an aspect of ovarian morphology that directly affects egg production and reproductive capacity in *Drosophila*.

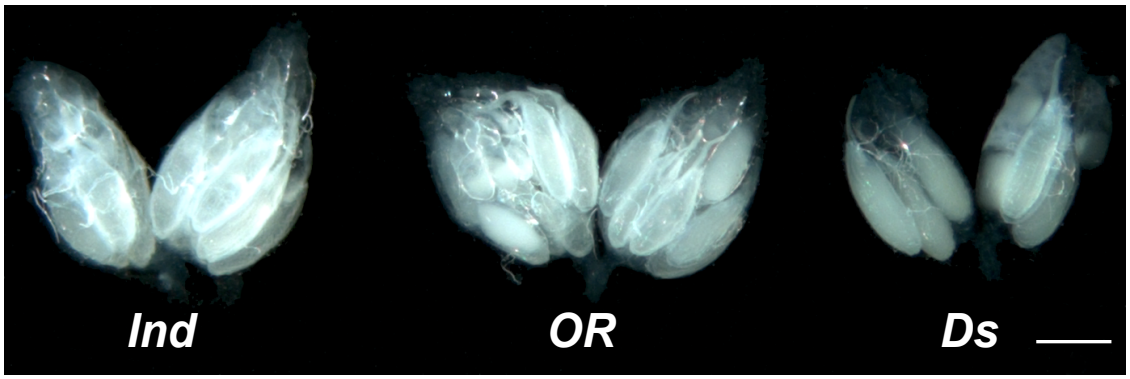
All insect ovaries are composed of ovarioles, which are egg-producing substructures of the ovary. Ovariole number is positively correlated with egg production and fecundity (), suggesting that this trait can have a significant impact on fitness and is likely to be under selective pressure. Ovariole number also varies across latitudinal (; David and Bocquet, 1975; Delpuech et al., 1995; Gibert et al., 2004; Paaby et al., 2010; Schmidt et al., 2005) and altitudinal (Collinge et al., 2006; Wayne et al., 2005) clines, further suggesting that this trait may be locally adaptive. Ovariole number variation across insects is dramatic, ranging from fewer than five per ovary in some flies to hundreds per ovary in crickets and grasshoppers (Büning, 1994).

Ovariole number has been the subject of extensive ecological and quantitative genetic studies for decades (reviewed in Hodin, 2009). Albeit not to single-gene resolution, these investigations have shown that ovariole number is a polygenic trait (Coyne et al., 1991; Thomas-Orillard, 1976), and inter- and intraspecific ovariole number variation is linked to changes at numerous loci (Bergland et al., 2008; Orgogozo et al., 2006; Telonis-Scott et al., 2005; Wayne et al., 2001; Wayne and McIntyre, 2002). Determining promising candidate genes from these QTL studies is difficult, because ovarian morphogenesis is relatively poorly understood, and only a small number of genes have been shown to play a specific role in ovariole formation (Gancz et al., 2011; Godt

and Laski; Hodin and Riddiford). Thus candidate genes within these loci have not yet been functionally investigated for causal links to ovariole number. We hypothesized that a better understanding of the cellular and developmental mechanisms governing ovariole formation would help to identify candidate genes that may underlie ovariole number evolution.

Ovariole morphogenesis begins with the formation of stacks of somatic cells, called terminal filaments (TFs), in the anterior of the larval ovary (Godt and Laski, 1995) (Figure 2A). Each TF is the starting point for the development of one ovariole, such that ovariole number is directly determined by TF number (Hodin and Riddiford, 2000). We previously showed that a major determinant of TF number is the total number of TF precursor cells present in the larval ovary before TF formation begins, and that TF cell number varies between *Drosophila* species with different ovariole numbers (Sarikaya et al., 2012). Here we explore even earlier developmental processes to understand why *Drosophila* lineages have different TF cell numbers, and whether TF cell number variation can explain differences in ovariole number in a broader range of Drosophilids. In this study we analyze and compare the process of ovarian morphogenesis in *Drosophila* lineages that independently evolved a significantly lower average ovariole number than the North American *D. melanogaster Oregon R* (*OR*) strain: the *D. melanogaster* "India" strain (*Ind*) and the single-niche specialist *D. sechellia* "Robertson" strain (*Ds*) (Figures 2.1, 2.2) (Markow and O'Grady, 2007). One hypothesis for the adaptive significance of lower ovariole number may be its positive correlation with larger egg size that often accompanies ecological specialization in *Drosophila* and other flies (Kambysellis et al., 1995; Markow et al., 2009; R´kha et al., 1997), and could potentially

lead to higher hatching rates or larval fitness (Azevedo et al., 1997). We show that similar TF cell numbers and therefore similar ovariole numbers are achieved in these lineages by changes in very different developmental processes. Establishing a smaller pool of somatic gonad cells during embryogenesis in *Ds*, or changing morphogenesis of specific ovarian cell types during larval development in *Ind*, both result in lower ovariole numbers than in *OR*. By analyzing the development of different ovarian cell types in these lineages, we demonstrate that within the same organ, evolutionary changes occur independently in different cell types. We use functional analysis in *D. melanogaster* to show that different genetic pathways influence these distinct developmental mechanisms. Our results show that major changes in reproductive capacity can evolve via distinct developmental mechanisms among closely related lineages.



	Ind	OR	Ds
Average Ovariole Number (per female)	24.8	36.4	15.2

Figure 2.1: Reduced ovariole number has evolved independently in *Ds* and *Ind*.

Adult ovaries and average ovariole number per female of *D. melanogaster* India (*Ind*) and Oregon R (*OR*) strains, and of *D. sechellia* (*Ds*). Scale bar = 0.5 mm.

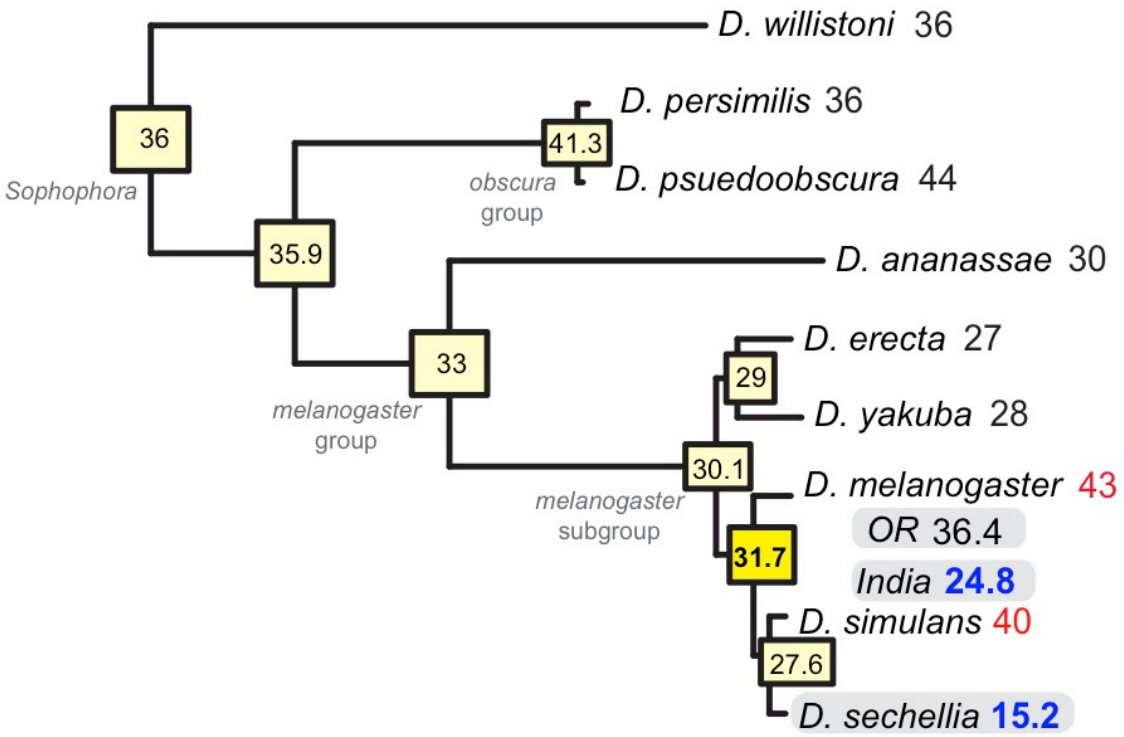


Figure 2.2: Ancestral state reconstruction of ovariole number across members of the genus *Drosophila*. Maximum likelihood values are indicated in boxes at nodes. Lineages analyzed in this study are highlighted in grey. The node representing the last common ancestor of *OR*, *Ind*, and *Ds* is bolded. Significant increases or decreases in average ovariole number relative to ancestral values are indicated in red and blue respectively. For 95% confidence intervals see Table 2.1.

Materials and Methods

Drosophila strains and mutant stocks.

D. melanogaster OregonR-C (Bloomington *Drosophila* Stock Center (BDSC) #5), *D. yakuba* (*Drosophila* Species Stock Center (DSSC) #1402–0261.01) and *D. sechellia* Robertson strain (DSSC #14021-0248.25) were obtained from the Hartl lab (Harvard U.). North American *D. melanogaster* strains (isofemale lines derived from females collected in respective locations) obtained from the DePace lab (Harvard Medical School) were Nevada-04 (*NV*), Raleigh-201 (*NC*), Catalina Island (*CA*), and Sante Fe (*NM*). The *D. melanogaster India* (DSSC #14021-0231.06) and *France* strains were a gift of the Ludwig lab (U. of Chicago). Other BDSC stocks used were the hypomorphic *InR* alleles InR^{E19} (#9646) and InR^{GC25} (#9554) and *w; P{w[+mW.hs]=GawB}bab1*$^{Pgal4\text{-}2}$*/TM6B Tb*^{1} (referred to as *babGAL4* in the text; #6803) (Cabrera et al., 2002).The *bab2* RNAi line *w; bab2-RNAi* (Transformant ID #49042) was obtained from the Vienna *Drosophila* RNAi Center.

Ancestral state reconstruction

Maximum likelihood estimates of ancestral character state and associated 95% confidence intervals at each internal node were derived using the Analysis of Phylogenetics and Evolution (APE) package in R (Paradis et al., 2004). The phylogenetic tree and branch lengths, derived from synonymous substitution rates in 12 Drosophilids, are from Heger and Ponting (2007). The ovariole numbers used in these analyses are from Markow et al. (2009).

Culture conditions and larval staging

Drosophila stocks were maintained at 25ºC at 60% humidity under optimal nutrition and without crowding as previously described (Sarikaya et al., 2012). For larval staging analyses, eggs were collected overnight on medium (supplemented with a 1cm^2 piece of filter paper soaked in N-caprylic acid (Sigma) for *D. sechellia*) in 6 cm-dish collection chambers. 18-22 hours after collection start, dishes were cleared of adult flies and hatched larvae. Newly hatched larvae were collected 2 hours after clearing and transferred to fresh vials containing standard medium (<100 larvae per vial), establishing L0 (±1 hr) larvae. At each time point, body size was used to guide selection of appropriately developed larvae. Larval-pupal transition (LP) stage larvae were identified as previously described (Ashburner et al., 2005).

Adult analysis: ovariole number and body size

Adult ovariole number was determined as previously described (Sarikaya et al., 2012). Adult tibia length was used as a proxy for adult body size (Macdonald and Goldstein, 1999). Images were taken using a Zeiss AxioImager Z1 and a Zeiss MRm AxioCam driven by AxioVision v4.6. Measurements were performed as previously described using Image J (v.1.45) software.

Larval analysis: TFC Number per TF, TFC Number, and TFC Size

These parameters were determined as previously described (Sarikaya et al., 2012) with the modification that optical confocal sections were captured at 0.9-1.2x zoom in 0.5µm thick sections spanning the entire ovary, and analyzed using Image J (v.1.45)

software. When reporting LP stage somatic cell proportioning, 'TF cells' measurements include a small proportion of cells that will adopt cap cell fate. Cap cell number per niche/TF averages 2.5 (Godt and Laski, 1995) and is constant among all lineages observed (not shown). Statistical comparisons between samples were made using a two-tailed Student's t-test.

Larval analysis: total cell number

Total cell number was counted using a similar methodology as TFC number counts. At the LP stage, "anterior somatic cells" are somatic cells located anterior to the germ cells, and include TFCs and apical cells, which were distinguished by the presence (TFC) or absence (apical) of Engrailed expression. In a few cases, cells adjacent to germ cells were also counted as anterior (apical) somatic cells if their nuclei were elongated along the A-P axis, as these cells are apical cells that are migrating posteriorly to delineate individual ovarioles. All other somatic cells were called "posterior somatic cells," the majority of which result from swarm cell migration, which is nearly completed by the LP stage. Swarm cells prior to late-third instar stages were identified by morphology and location within the ovary relative to other cell types. Germ cells were identified by Vasa expression.

Larval analysis: ovary volume

Ovary volume was approximated by measuring the volume of all ovarian nuclei using Volocity (v.4, Perkin Elmer) to define "objects" as those points exceeding 7.5% intensity level (empirically determined to be the optimal intensity value) in the Hoechst

channel; objects smaller than 10μm^3 were discarded. The largest object identified was recorded as the ovary volume approximation. The volumes of additional objects were added to the largest volume if the object was >1% the volume of the largest object.

Immunohistochemistry

Immunostaining was carried out as previously described (Sarikaya et al., 2012). The following primary antibodies were used: mouse 4D9 anti-Engrailed (1:40, Developmental Studies Hybridoma Bank), guinea pig anti-Traffic jam (1:30,000, gift of D. Godt, U. of Toronto), rabbit anti-Vasa (1:500, gift of P. Lasko, McGill U.). Secondary reagents used were Hoechst 33342 (Sigma, 1:500 of 10 mg/ml stock solution), goat anti-mouse Alexa 568, goat anti-guinea pig Alexa 488, and donkey anti-rabbit Alexa 647 (1:500, Invitrogen). Samples were mounted in Vectashield (Vector labs) and imaged using a Zeiss LSM 710 confocal microscope.

Results

Reduced ovariole number convergently evolved in Ds *and* Ind.

Ovariole number is highly variable among the Drosophilids (reviewed by Hodin, 2009). Although ovariole number is phenotypically plastic and can vary due to different environmental or nutritional conditions (Capy et al., 1993; Kambysellis and Heed, 1971; Sarikaya et al., 2012), under constant environmental conditions it falls within a heritable, species-specific range. For this study we chose to analyze two strains with a significantly lower average ovariole number than *D. melanogaster* Oregon R (*OR*). The *India* (*Ind*) strain of *D. melanogaster* has an average of 24.8 ovarioles per female, while *D. sechellia*

(*Ds*) has an average of 15.2 ovarioles per female, both of which are significantly lower than the *OR* average of 36.4 ovarioles per female (Figure 2.1). *Ind* likely shared a last common ancestor with *OR* in Africa prior to human commensal dispersal in the Neolithic (Capy et al., 2004). *Ds* diverged from the lineage containing *D. melanogaster* approximately 5.4 million years ago (Tamura et al., 2004), and has evolved a single-niche specialization on the *Morinda citrifolia* fruit as its plant host in the Seychelles (R´kha et al., 1997). Given the relatively higher ovariole numbers observed in most other members of the *melanogaster* subgroup (Figure 2.2), we therefore hypothesized that the reduction in ovariole number had occurred independently in the *Ind* and *Ds* lineages. To test this hypothesis, we performed an ancestral state reconstruction for ovariole number across the *Drosophila* family to generate a prediction for the ovariole number in the ancestor to *OR*, *Ind* and *Ds*. The maximum likelihood estimate for the average ovariole number of the ancestor to *OR*, *Ind*, and *Ds* is 31.7 ovarioles per female, with a 95% confidence interval of 25.2-38.3 ovarioles (Figure 2.2, Table 2.1). Average ovariole number per female in *OR* (36.4) is within this range, indicating that ovariole number in *OR* is not significantly different from the number hypothesized for its shared ancestor with *Ind* and *Ds*. However, average ovariole number in both *Ds* (15.2) and *Ind* (24.8) are below the ancestral range, indicating that ovariole number was independently reduced in both of these lineages. To address the possibility that *Ind* represents a segregating variant of the North American *D. melanogaster* range, we note that ovariole numbers in Indian populations (Rajpurohit et al., 2008) are, on average, smaller than those in North American populations (Capy et al., 1993). We also counted ovariole number in four additional *D. melanogaster* strains from North America, and found that their average ovariole numbers were always higher than

those for *Ind* (not shown). Taken together, these data show that ovariole number in *OR* is similar to the ancestral state of these three lineages, and reduced ovariole number convergently evolved in *Ind* and *Ds*.

Table 2.1: Confidence intervals for strains in this report.

Genotype	Adult Ovariole Number per ovary (95%CI)
Ind	12.2 (0.60)
OR	18.2 (0.87)
Ds	7.6 (0.27)
Nevada-04 (*NV*)	18.4 (0.93)
Raleigh-301 (*NC*)	22.1 (0.95)
Catalina Island (*CA*)	22.6 (0.98)
Santa Fe (*NM*)	22.5 (0.90)

Table 2.1: Confidence intervals for strains in this report. 95% confidence intervals for indicated average ovariole numbers (per female) derived from ancestral state reconstruction analysis (Figure 2.2). Nodes are indicated either by group name or by brackets containing the relevant descendant groups or species.

TF cell number at the larval-pupal transition stage determines adult ovariole number in Drosophila.

Ovariole morphogenesis depends on the proliferation and differentiation of somatic gonad cells during early larval stages, and subsequent terminal filament (TF) formation during later larval stages (Figure 2.3). We previously showed that adult ovariole number difference between the cosmopolitan species *D. melanogaster* and *D. yakuba* is correlated with differences in the number of a specific ovarian cell type, TF cells, at the LP stage (Sarikaya et al., 2012). Here we asked whether a difference in TF cell number also explained ovariole number differences in intraspecies and ecological specialist species comparisons. We found that in both *Ind* and *Ds*, TF number at the LP stage determines adult ovariole number (Figure 2.4). Previous studies had suggested that TF cell number per terminal filament or TF cell size might influence TF number and thus ovariole number (Hodin and Riddiford, 2000). We examined both of these parameters and found that neither was sufficient to account for ovariole number differences between the lineages (Figure 2.5). These data indicate that TF cell number at the LP stage is a robust predictor of ovariole number within and between *Drosophila* species, and also among *Drosophila* species that occupy varying ecological niches (Figure 2.4B). We also tested the hypotheses that the TF cell number variation between these lineages was due to overall growth differences of the entire fly or of the ovary, or to differences in germ cell number. We found that neither hypothesis was supported. Neither germ cell number at any pre-LP stage of development (Figure 2.6) nor ovary size (Figure 2.7) nor body size (Figure 2.8) was significantly correlated with adult ovariole number or TF cell number. The *Ds* ovary is significantly smaller than the *OR* ovary (Figure 2.7B) and contains fewer

TF cells (Figure 2.5B). However, the *Ind* ovary is slightly bigger than the *OR* ovary (Figure 2.7B) yet has significantly fewer TF cells (Figure 2.5B). This suggests that in *Drosophila*, specific mechanisms exist for precise control of TF cell number, leading to lineage-specific ovariole number.

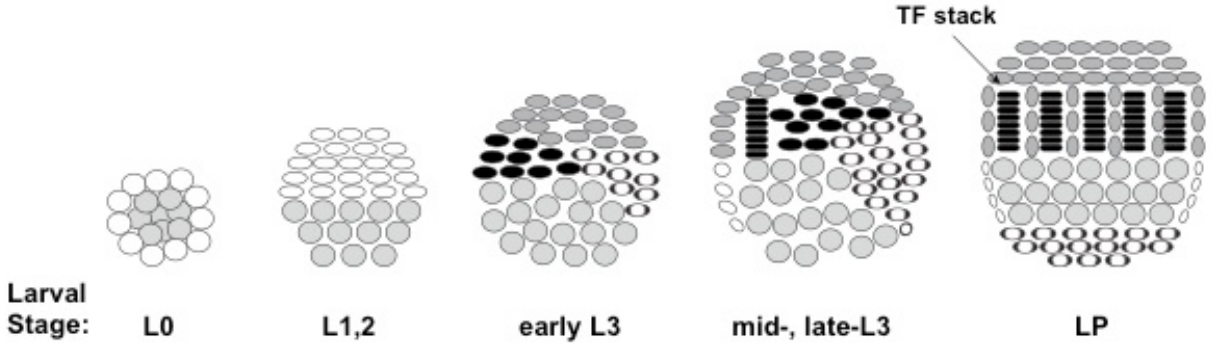


Figure 2.3: Schematic of ovary development and ovariole formation. See text for detailed description.

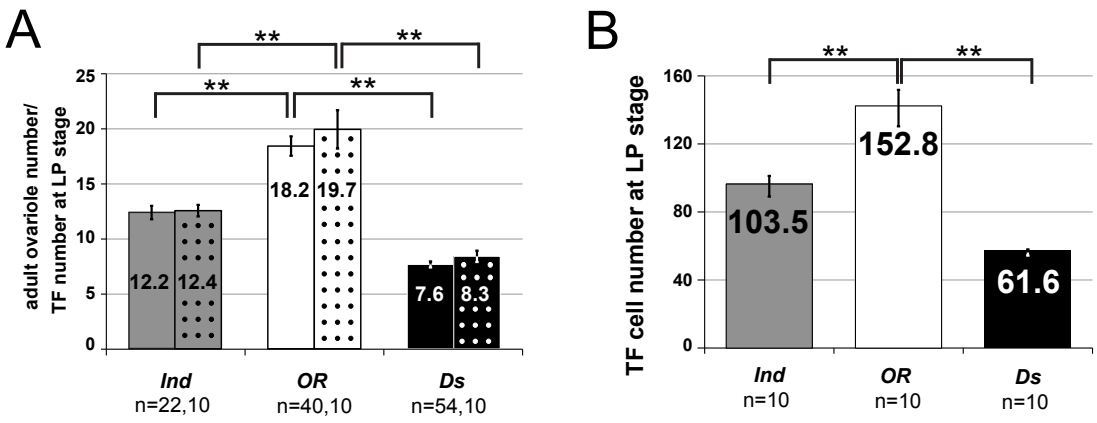


Figure 2.4: Reduced ovariole number within and between *Drosophila* species is a result of reduced TF cell number. (A) Mean adult ovariole number (solid bars) and mean LP stage terminal filament (TF) number (stippled bars) in all three lineages. (B) Mean TF cell number at LP stage in all three lineages. In A-B, *n*=number of ovaries analyzed (in A, n=*x*, *y* are number of ovaries analyzed for ovariole number (*x*) and TF number measurements (*y*) respectively), error bars show 95% confidence interval, ** $p<0.001$.

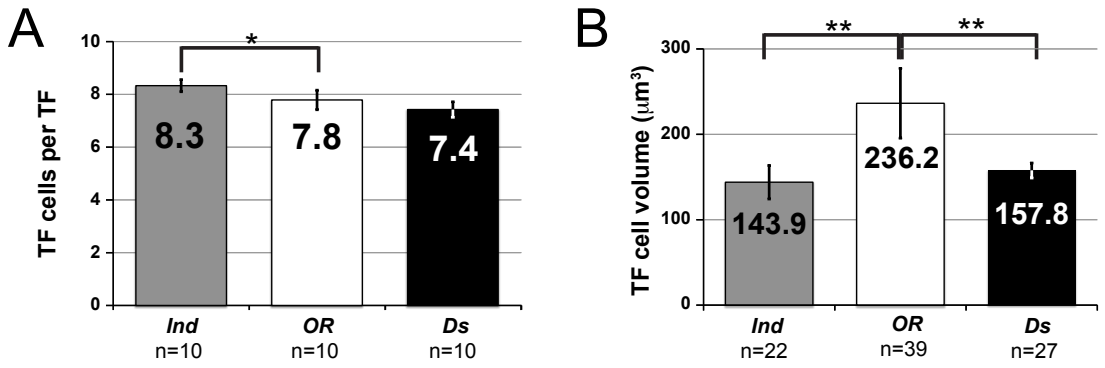


Figure 2.5: Reduced ovariole number between *Drosophila* lineages is not due to changes in TF morphogenesis or cell size. (A) TF cell number per TF for *Ind*, *OR* and *Ds*. (B) TF cell size for all three lineages. * $p<0.05$; ** $p<0.001$. Error bars show 95% confidence interval.

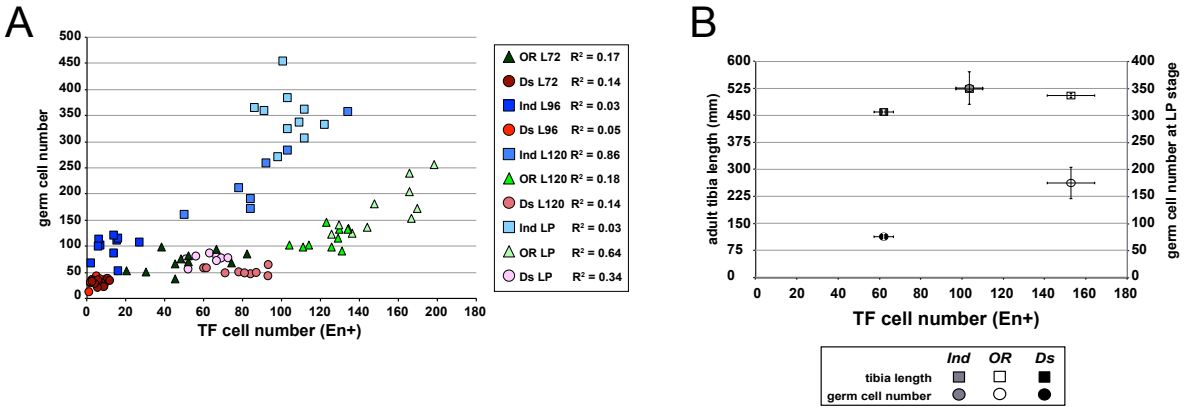


Figure 2.6: Germ cell number does not predict TFC number. (A) Number of germ cells and TF cells from L0 to LP stage in *OR*, *Ind* and *Ds*. Each point shows germ cell and TF cell counts from a single individual. (B) TF cell number does not vary predictably with adult tibia length (squares, left axis), a proxy for body size, or LP stage germ cell number (circles, right axis).

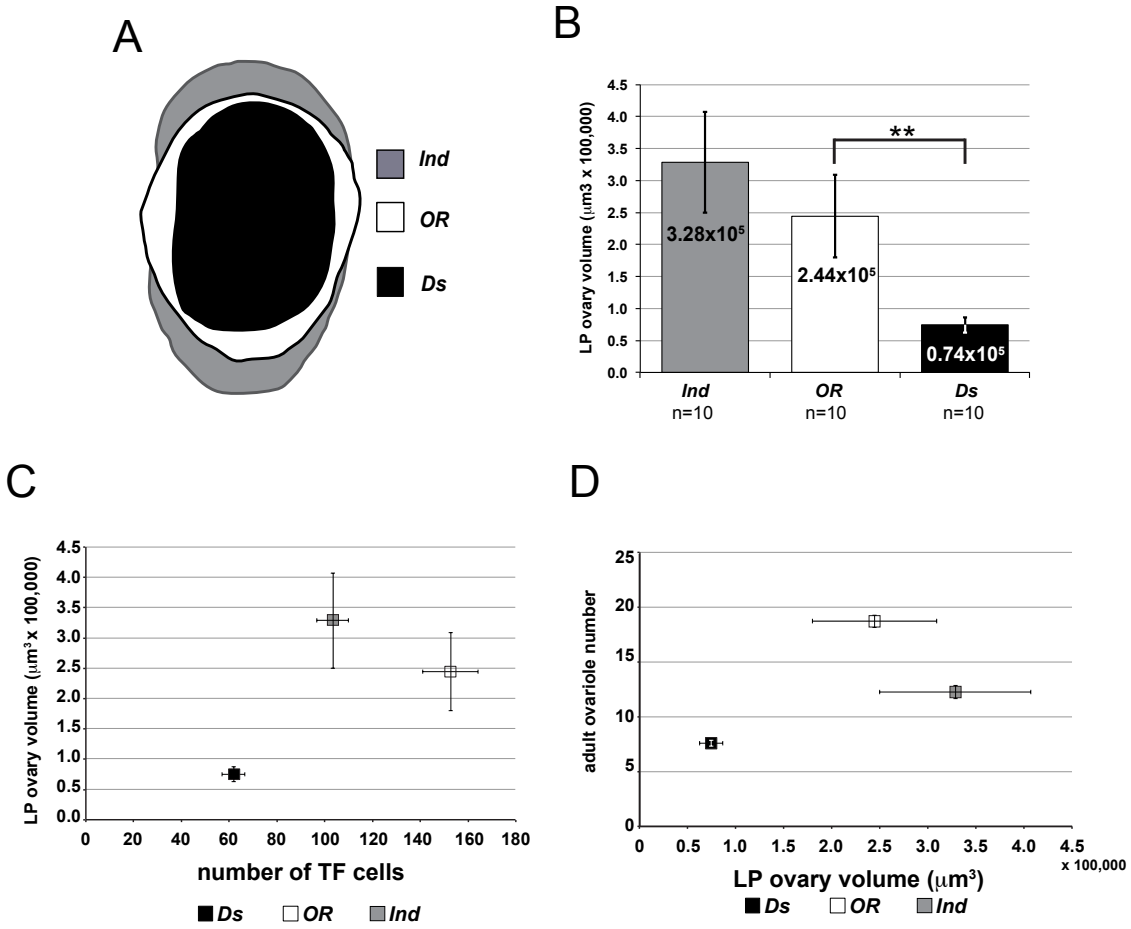


Figure 2.7: Larval ovary size does not predict TFC number. (A) Trace of optical cross-section through the widest point of an ovary of each lineage. (B) Mean larval ovary volumes for all three lineages. Error bars show 95% confidence interval, ** $p<0.001$. (C) LP stage ovary size does not vary predictably with TF cell number. (D) LP stage ovary size does not vary predictably with adult ovariole number.

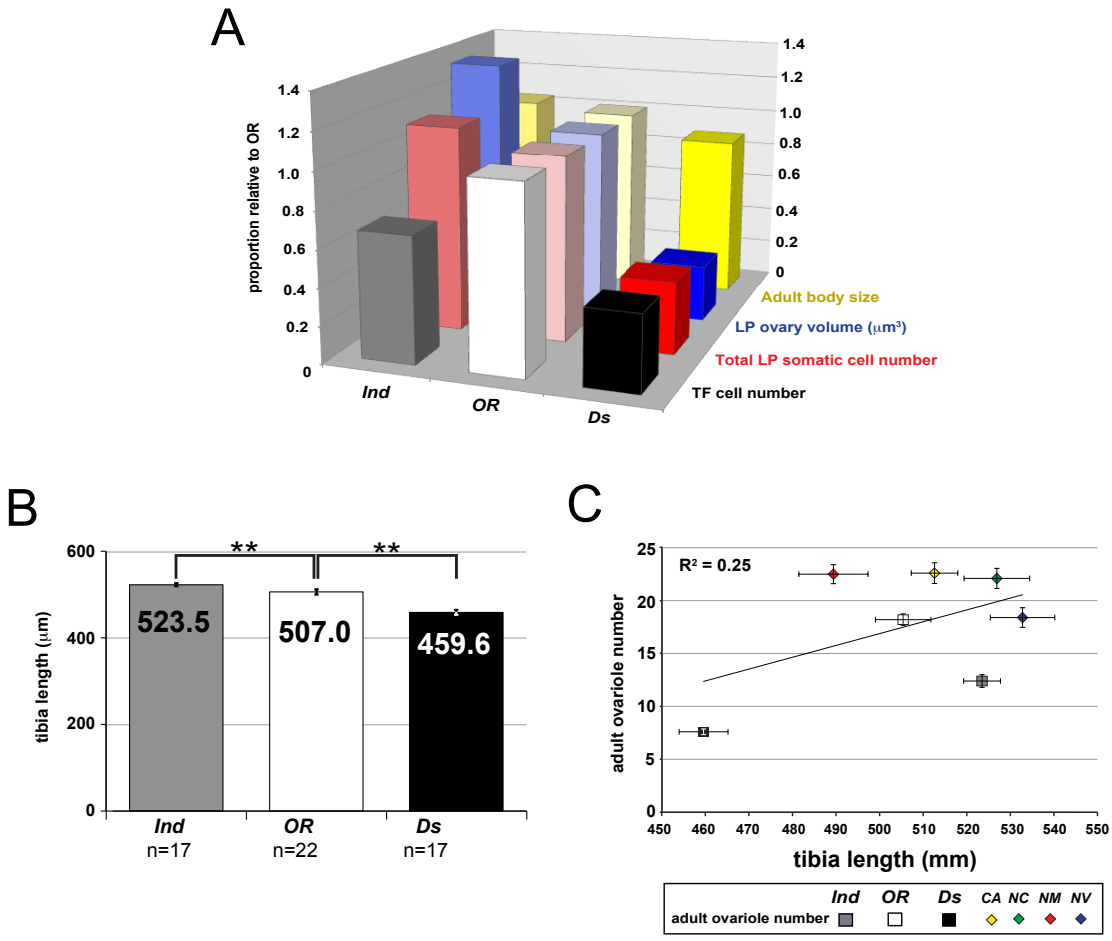


Figure 2.8: Adult body size does not predict TFC number. (A) Relative proportions of adult body size (yellow shades) LP stage ovary volume (blue shades), total ovarian somatic cell number (red shades) and TF cell number (grey/white/black bars) for all three lineages. For each parameter, value is normalized to the corresponding *OR* value. (B) Tibia length in all three lineages. ** $p<0.001$. (C) Tibia length is not correlated with adult ovariole number in *OR*, *Ind*, *Ds*, and four additional North American *D. melanogaster* strains.

A constant proportion of anterior somatic ovarian cells are specified as TF cells.

At the LP stage, somatic cells of the ovary lie both anterior and posterior to germ cells (Figure 2.3). TF cells are derived exclusively from the anterior cell population. We asked if the three *Drosophila* lineages specified different proportions of TF cells from anterior cells. We found that the total number of anterior cells is different among lineages, but across all three lineages, a similar proportion of anterior somatic cells differentiate into TF cells (Figure 2.9). This suggests that anterior somatic cell number is the key parameter that determines TF cell number. We therefore investigated the developmental origin of anterior cells, and whether decreased TF cell number in *Ind* and *Ds* compared to *OR* is a consequence of the same or different developmental mechanisms.

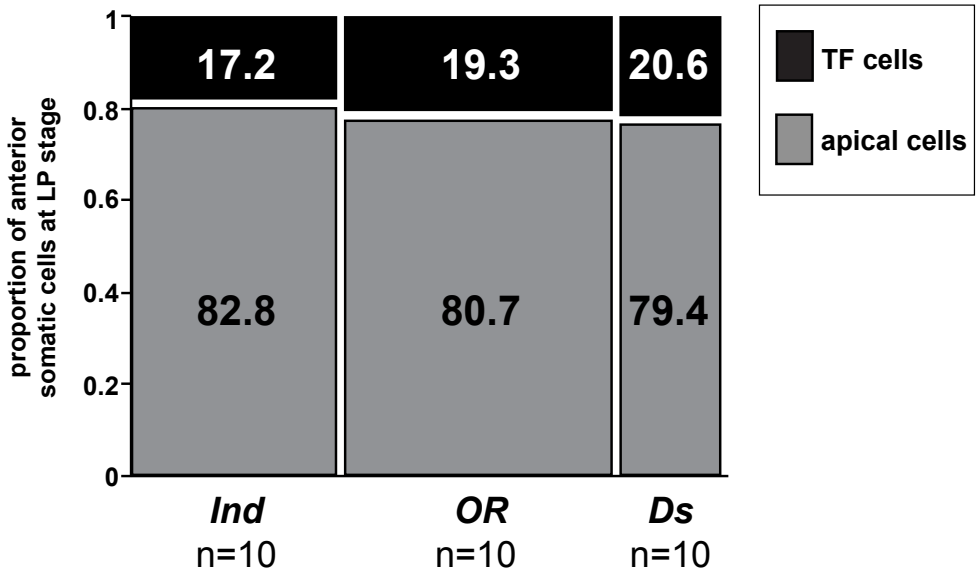


Figure 2.9: Anterior somatic cell number, and not germ cell number, predicts TF cell number in *OR*, *Ind*, and *Ds*. Mosaic plots of proportions of the two anterior cell types, TF cells (black) and apical cells (grey) at LP stage in all three lineages. Bar width is proportional to total cell number in a given lineage.

A reduced number of somatic gonad precursor cells established during embryogenesis leads to reduced TF cell number in Ds

Ovarian development begins during embryogenesis when a small number of somatic gonad precursor (SGP) cells are specified in the mesoderm of abdominal parasegments 10-12 (Boyle and DiNardo, 1995) (Figure 2.3). SGP cells undergo up to one mitotic division before hatching (L0), resulting in a small gonad primordium in the first larval instar. Somatic gonad cells proliferate and remain largely morphologically undifferentiated until later larval stages. During mid-third instar, a group of anterior somatic cells called "swarm" cells (Couderc et al., 2002) migrate laterally past the germ cell cluster towards the posterior of the ovary (Figure 2.3, Figure 2.10). Once they are posterior to the germ cells, somatic cells differentiate to form the interfollicular stalk, basal stalk, and basal cells in later larval and pupal development (Couderc et al., 2002). A subset of the cells that remain anterior to the germ cells express Engrailed and become TF cells (Bolívar et al., 2006; Godt and Laski, 1995). By the LP stage, anterior somatic ovary cells are thus divided into two cell populations: cells that express Engrailed (TF and cap cells), and those that do not (apical cells, which will migrate posteriorly between TF stacks to delineate individual ovarioles). We counted the number of gonadal cell types throughout ovary development in *OR*, *Ind*, and *Ds*. The number of SGP cells in *Ds* L0 larvae is significantly smaller than in *OR* ($p<0.001$; Figure 2.11, Figure 2.12A, A'), and the pool of somatic gonad cells remains comparatively smaller throughout development (Figure 2.12A). Importantly, this difference is specific to the somatic gonad and does not reflect a reduction in primordial ovary size as a whole, as L0 germ cell number is similar across all three lineages at this stage (Figures 2.12B, B'). As a result, *Ds* has a reduced

number of all somatic cell types, including TF cells, at the LP stage ($p<0.001$; Figure 2.13). The same proportion of "swarm" cells migrate to the posterior in both *OR* and *Ds* (Figure 2.14). Taken together, these data show that the developmental basis of evolutionary reduction in *Ds* ovariole number is primarily a change in the number of SGP cells initially established during embryogenesis.

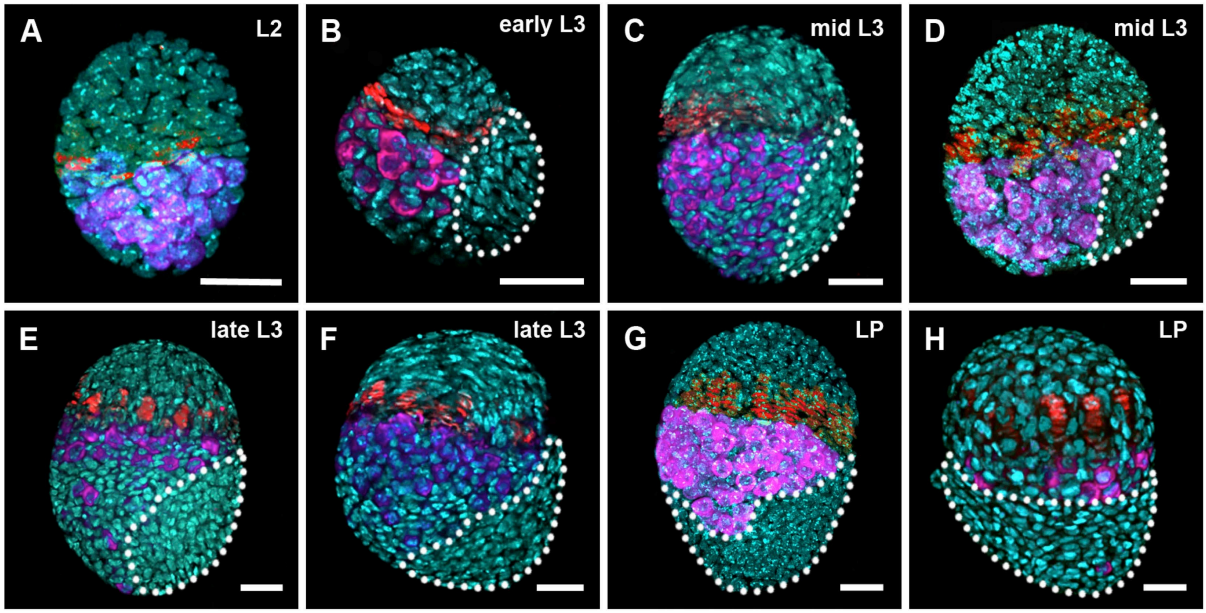


Figure 2.10: Swarm cell migration in late larval life. (A) Ovaries of *OR* at L2 stage. Most somatic cells are still located anterior to the germ cells. (B) In early L3, swarm cells begin migration lateral to the germ cells towards the posterior. (C, D) As the L3 stage progresses swarm cells migrate towards the posterior. (E, F) during late L3 stages, swarm cells complete their movement to lie posterior to the germ cells. (G, H) By late LP stage, swarm cell migration has completed and the migrating cells lie entirely posterior to the germ cells. In all panels, white dashed line demarcates "swarm" cells, anterior is up, scale bar = 20 μm. All panels show 3D reconstructions of optical sections, except for A, D and G, which show maximum projections of optical sections.

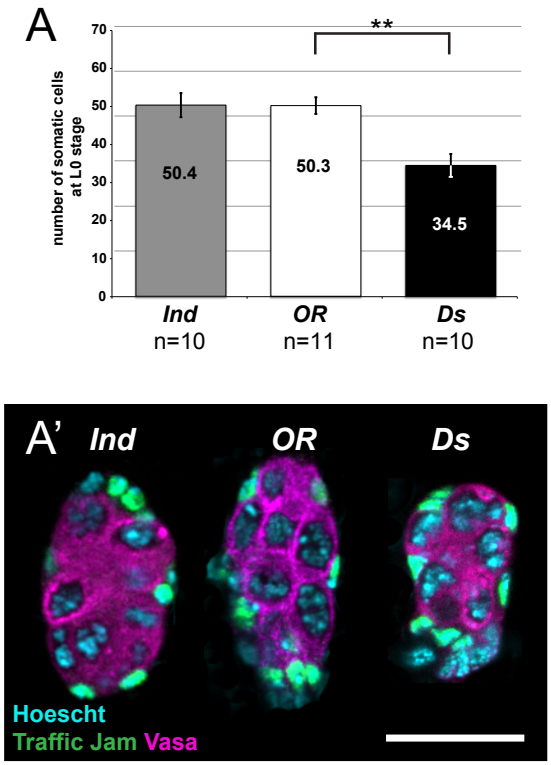


Figure 2.11: Ovariole number reduction results from changes in the number of somatic gonad precursors in *Ds*, and from changes in somatic cell type segregation in the larval ovary in *Ind*. (A) Mean somatic ovarian cell number at first larval instar (L0) 0-3 hours after hatching (h AH) in all three lineages. (A') Optical sections of L0 ovaries. Scale bar is 20 μm. ** $p<0.001$. Error bars show 95% confidence interval.

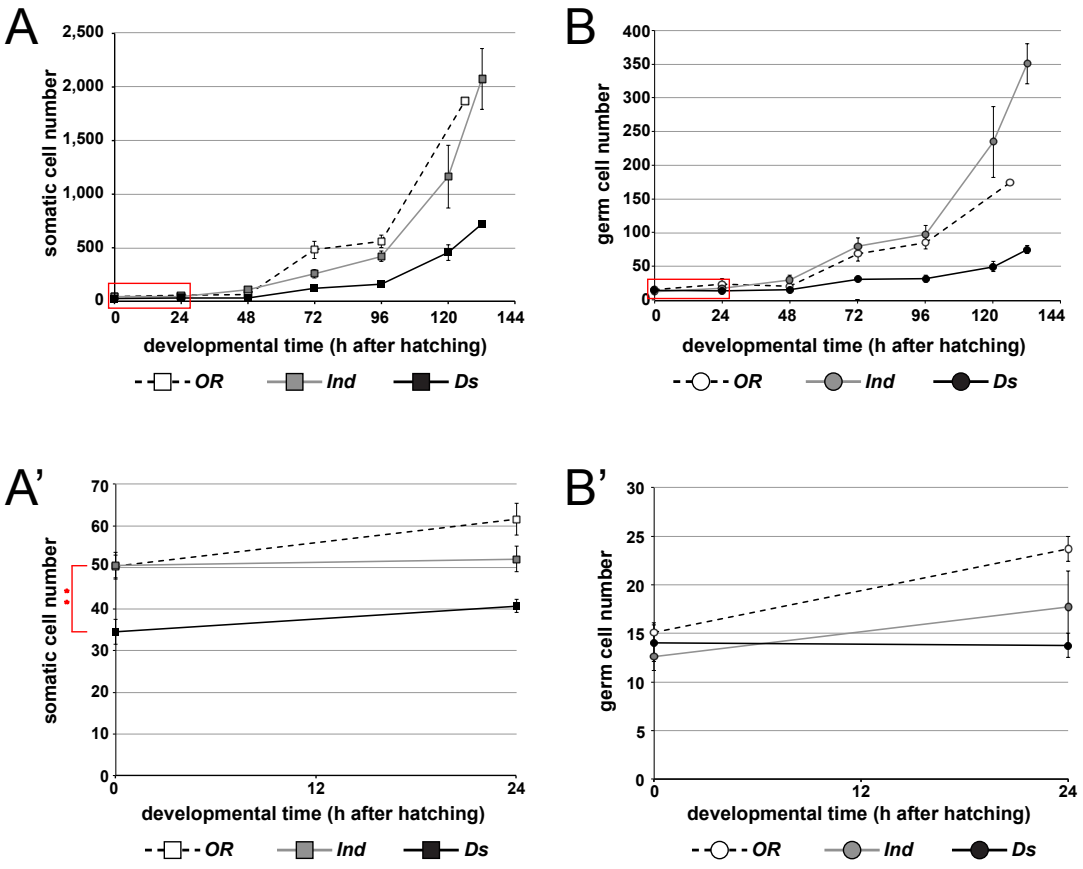


Figure 2.12: Ovarian cell proliferation throughout larval life and TF cell allocation at LP stage. (A) Number of total ovarian somatic cells from L0 to LP stage in *OR*, *Ind* and *Ds*. (A') Close up view of the region demarcated by the red box in (C), showing a significant difference in SGP cell number at L0 between *Ind* and both *D. melanogaster* strains. (B) Number of total germ cells from L0 to LP stage in *OR*, *Ind* and *Ds*. (B') Close up view of the region demarcated by the red box in (D), showing no significant difference in germ cell number at L0 between any of the three lineages.

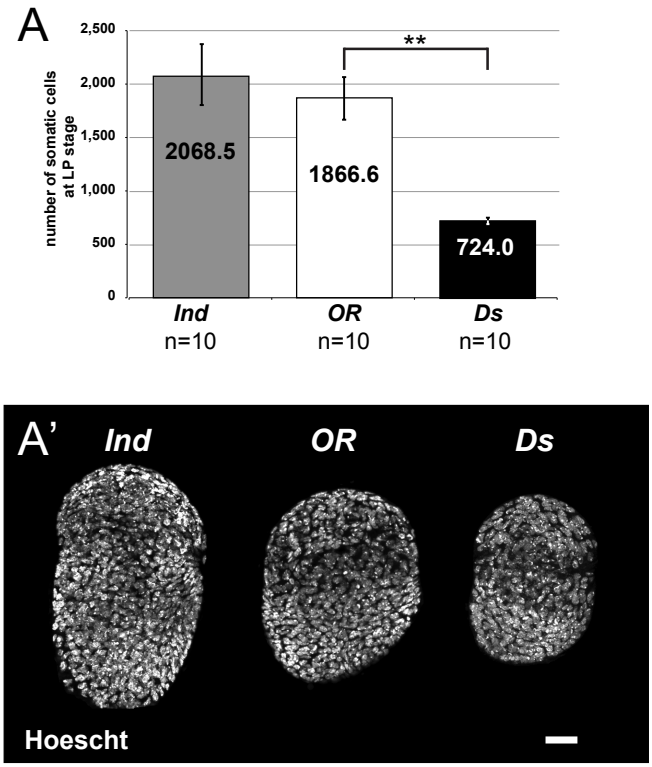


Figure 2.13: Ovariole number reduction results from changes in the number of somatic gonad precursors in *Ds*, and from changes in somatic cell type segregation in the larval ovary in *Ind*. (A) Mean total ovarian somatic cell number at LP stage in all three lineages. (A') Maximum projections of optical sections of LP stage ovaries of *Ind*, *OR* and *Ds*. Anterior is up in A'. Scale bar is 20 μm. ** $p<0.001$. Error bars show 95% confidence interval.

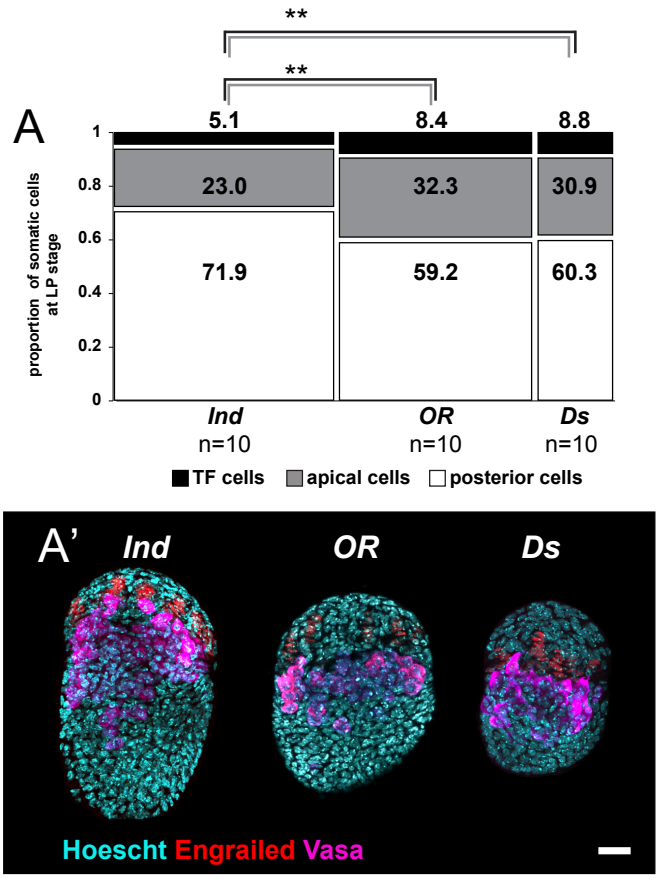


Figure 2.14: Ovariole number reduction results from changes in the number of somatic gonad precursors in *Ds*, and from changes in somatic cell type segregation in the larval ovary in *Ind*. (A) Mosaic plots of the proportions of the three somatic cell types at LP stage (TF cells (black), apical cells (grey), and posterior cells (white)). Bar width is proportional to total cell number in a given lineage. (A') Same images as in B' but with cell populations distinguished by gene expression. TF cell values include a small proportion of cells that will become cap cells; cap cell number per TF is constant among all lineages observed (not shown). Intermingled cells (Li et al., 2003) are classified here as posterior cells; their number regulates germ cell number (Gilboa and Lehmann, 2006).

Anterior is up in A'. Scale bar is 20 μm. ** $p<0.001$. Error bars show 95% confidence interval.

Changes in ovarian morphogenesis during late larval stages lead to reduced TF cell number in Ind

In contrast to what we observed in *Ds*, SGP cell number is not significantly different between *Ind* and *OR* ($p=0.95$; Figure 2.11, Figure 2.12A, A'). Somatic cell proliferation rates between *Ind* and *OR* are similar (Figure 2.12A), and both lineages reach similar numbers of total somatic cells by the LP stage ($p=0.27$, Figure 2.13). We therefore examined swarm cell migration and anterior/posterior somatic cell allocation in these ovaries. We found that significantly more swarm cells migrate to the posterior of the ovary in *Ind* than in *OR* (Figure 2.14). As a consequence, a significantly smaller proportion of cells are allocated to anterior cell fates in *Ind* than in *OR* (Figure 2.14). Because the same proportion of anterior cells become TF cells in these strains (Figure 2.9), we conclude that differences in swarm cell migration cause the observed reduction in *Ind* TF cell number relative to *OR*. These data indicate that *Ind* ovariole number reduction proceeds through different developmental mechanisms than those operating in *Ds*: rather than a difference in embryonic SGP cell establishment, in *Ind* descendants of the same number of SGP cells are allocated to specific cell fates in dramatically different ways. Notably, the variations in ovarian development occur at very different stages in *Ds* and *Ind*, but the final effect on TF cell number is nonetheless the same.

Loss of bab2 *function in* D. melanogaster *reduces TF cell number by affecting ovarian morphogenesis during larval stages*

Because these two developmental events occur at different developmental times and involve distinct cellular behaviors, we hypothesized that different genetic mechanisms could direct these developmental processes independently of one another. Quantitative genetics approaches to ovariole number variation have implicated different loci linked to interspecies (Coyne et al., 1991; Orgogozo et al., 2006) and intraspecies (Bergland et al., 2008; Wayne et al., 2001; Wayne and McIntyre, 2002) variation. However, few candidate genes have been suggested and none of the genes contained in these loci have yet been tested functionally for a role in ovariole number. We therefore revisited these data in light of our new developmental data on the differences between ovarian development in *Ind*, *Ds* and *OR*.

We first looked for candidate loci that might play a role in ovarian morphogenesis, specifically swarm cell migration. A QTL study examining ovariole number in recombinant inbred lines of *D. melanogaster* identified a major effect locus that contains the *bric-á-brac* locus, which encodes for two genes *bab1* and *bab2* (Bergland et al., 2008; Couderc et al., 2002). Both genes are expressed in the late larval ovary, are not expressed in the embryonic gonad, and play a role in ovarian morphogenesis (Godt and Laski, 1995; Sahut-Barnola et al., 1995). Because *bab2* is highly expressed in swarm cells at the time of their migration (Couderc et al., 2002), we hypothesized that specifically reducing *bab2* function in the somatic ovary might affect cell migration behavior and consequently TF cell number. We used the *bab:GAL4* driver (Cabrera et al., 2002), which is expressed in somatic cells of the larval ovary and most strongly in anterior somatic cells, to knock down *bab2* function in these cells via RNAi (Figure 2.15A). *bab:GAL4>>bab2*^{RNAi} did not alter the number of SGP cells specified relative to controls, *OR*, or *Ind* (p=0.37, 0.88,

0.85, respectively; Figure 2.15B). However, we found that swarm cell migration was abnormal in *bab:GAL4>>bab2*^{RNAi} ovaries: both the migration (Figure 2.15A) and the number (Figure 2.15C) of swarm cells were affected. We counted all cell types in these ovaries at the LP stage to quantify the effects on anterior/posterior somatic cell allocation, and found that *bab2* knockdown resulted in a significantly greater proportion of posterior cells at the expense of anterior cells (Figure 2.15C). Interestingly, average anterior/posterior proportions were nearly identical to those in *Ind* (Figures 2.14, 2.15C). The proportion of anterior cells that became TF cells was similar to all three wild type lineages and *bab2*^{RNAi};+ controls (p=0.13 (*Ind*), 0.40 (*OR*), 0.76 (*Ds*), 0.02, respectively; Figure 2.16A).

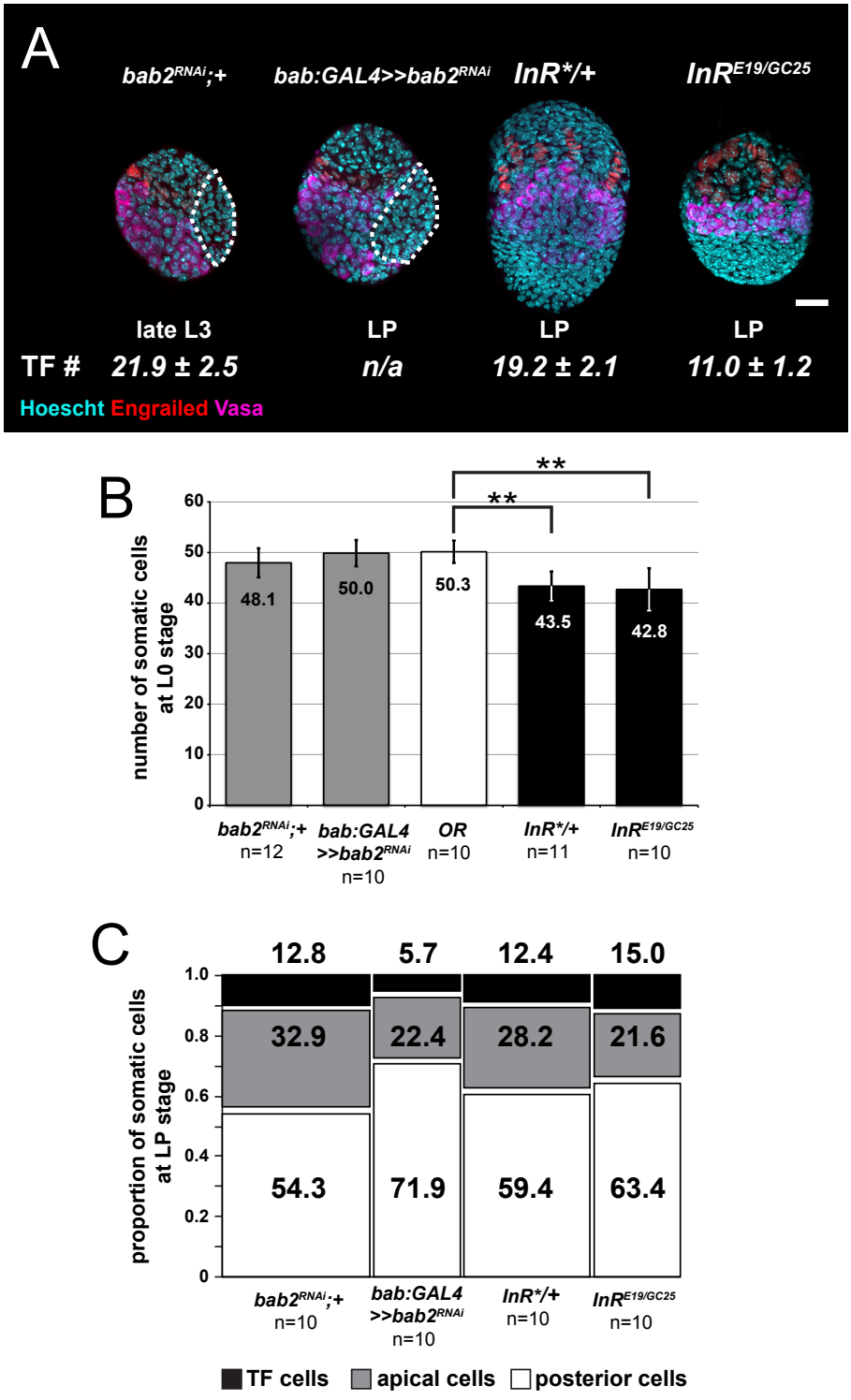


Figure 2.15: Distinct genetic mechanisms regulate ovariole number by altering SGP cell number changes or cell type allocation.

Figure 2.15 (Continued): Distinct genetic mechanisms regulate ovariole number by altering SGP cell number changes or cell type allocation. (A) Ovaries of *bab2^{RNAi};+*, *bab:GAL4>>bab2^{RNAi}*, *InR^{E19} or InR^{GC25}/TM3*, and *InR^{E19}/InR^{GC25}* at LP stage, except for *bab2^{RNAi};+*, shown at late-third instar to visualize swarm cell migration. All images are maximum projections of optical sections, anterior is up, scale bar = 20 μm. (B) Mean SGP cell number at L0 for *bab2^{RNAi};+*, *bab:GAL4>>bab2^{RNAi}*, *InR^{E19} or InR^{GC25}/TM3*, *InR^{E19}/InR^{GC25}* and *OR* ovaries. Error bars show 95% confidence interval. (C) Mosaic plots of proportions of somatic cell types at LP stage in genetic backgrounds shown in (A). Bar width is proportional to total cell number in a given lineage.

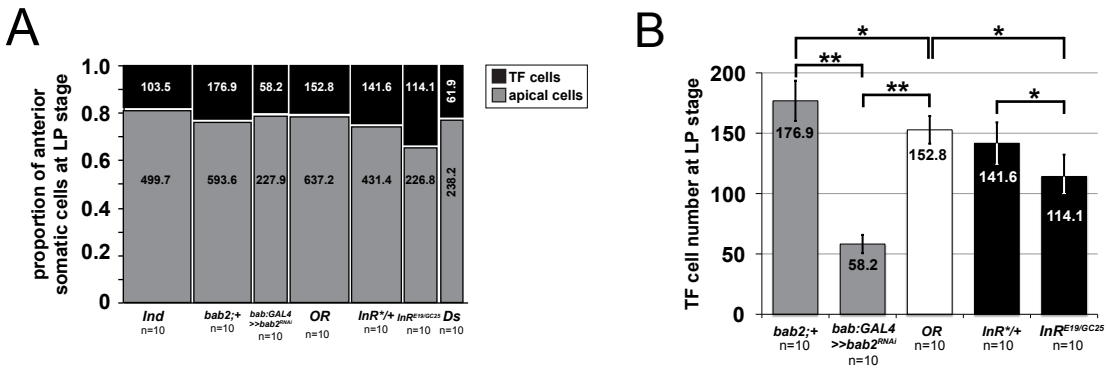


Figure 2.16: Effects of *InR* and *bab2* loss of function on LP stage TF cell number and TF cell specification from anterior somatic cells. (A) Mosaic plots of proportions of the two anterior cell types, TF cells (black) and apical cells (grey) at LP stage in loss of function conditions for both *InR* and *bab2* (see text and Figure 4 for details). Despite some modification of TF morphogenesis in $InR^{E19/GC25}$ LP stage ovaries (see Figure 2.17A), TF cell number is significantly reduced compared to controls (see Figure 2.17B). In A-B, * $p<0.05$; ** $p<0.001$, error bars show 95% confidence interval. (B) TF cell number at LP stage is significantly reduced by loss of function conditions for both *InR* and *bab2* (see text and Figure 2.14 for details).

Because *bab* also plays a role in the process of TF cell stacking to form TFs (Godt and Laski, 1995), *bab:GAL4>>bab2*^{RNAi} ovaries ultimately fail to make normal TFs or ovarioles. However, as a consequence of reduced anterior cell number, TF cell number was reduced in *bab:GAL4>>bab2*^{RNAi} ovaries compared to controls (Figure 2.16B), suggesting that TF number and adult ovariole number would also be reduced in these females. These results show that changes in *bab2* function can influence TF cell number by affecting swarm cell migration, thereby altering the anterior/posterior proportioning of somatic ovary cells. Importantly, body size was unchanged in *bab:GAL4>>bab2*^{RNAi} females compared to controls (p=0.64; Figure 2.17A), demonstrating that ovariole number can be changed independently of body size. These phenotypes mimic the critical developmental differences during larval development that underlie ovariole number differences between *OR* and *Ind*, while leaving SGP cell establishment in embryogenesis unaltered.

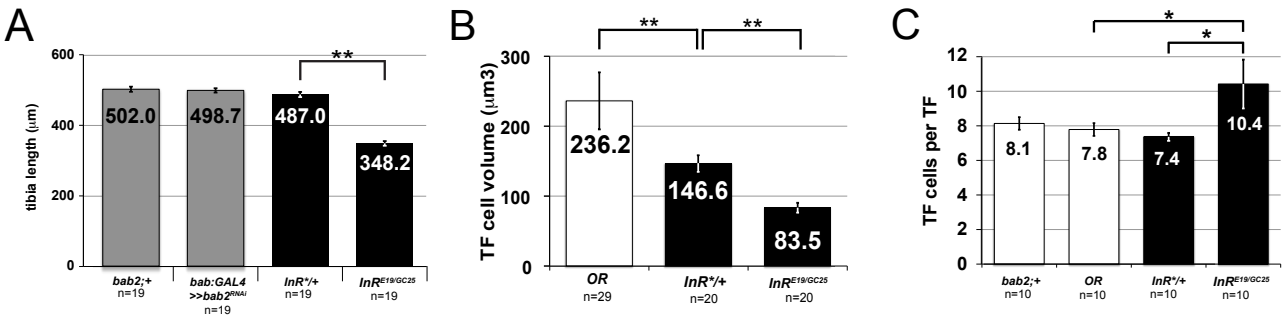


Figure 2.17: Effects of *InR* and *bab2* loss of function on TF cell size and TF morphogenesis. (A) Tibia length is not significantly different in *bab:Gal4>>bab2*^{RNAi} females compared to controls. Tibia length is significantly reduced in *InR*$^{E19/GC25}$ females compared to *InR* heterozygotes. (B) TF cell size is significantly reduced in LP stage ovaries of *InR*$^{E19/GC25}$.transheterozygotes and in heterozygotes of both loss-of-function alleles. (C) TF cell number per TF in LP stage ovaries for *InR*$^{E19/GC25}$, *InR* heterozygotes and *bab2*^{RNAi}*;+* controls does not differ significantly from *OR* (see Figure 2.5A). TF cell number per TF is not shown for *bab:Gal4>>bab2*^{RNAi} ovaries as these show abnormal TF stack formation (Godt and Laski, 1995). * $p<0.05$; ** $p<0.001$. Error bars show 95% confidence interval.

Loss of InR *function in* D. melanogaster *reduces TF cell number by affecting SGP cell establishment*

We next examined previous QTL analyses for genes that might affect TF cell number by affecting SGP cell number. The *Drosophila Insulin receptor* (*InR*) gene emerged as a top candidate for investigation. *InR* is contained within a large-effect locus linked to ovariole number difference between *D. simulans* and *D. sechellia* (Orgogozo et al., 2006). *InR* is the single insulin-like peptide receptor in *Drosophila* that mediates the insulin signaling pathway (Petruzzelli et al., 1986), a major regulator of cell proliferation and body size in animals (Goberdhan and Wilson, 2003). Reduced insulin signaling in *Drosophila* leads to reduced body size as a consequence of reductions in both cell number and cell size (Böhni et al., 1999; Chen et al., 1996; Shingleton et al., 2005), but patterning and morphogenesis programs remain intact. Moreover, loss-of-function mutants in the *InR* substrate *chico* have reduced adult ovariole number (Richard et al., 2005; Tu and Tatar, 2003).

We hypothesized that flies with reduced insulin signaling activity would have a lower adult ovariole number due to a reduced number of SGP cells specified during embryogenesis, thereby reducing TF cell number. We confirmed that $InR^{E19/GC25}$ loss-of-function trans-heterozygotes contain significantly fewer TFs ($p<0.001$; Figure 2.15A) and TF cells ($p<0.001$; Figure 2.16B) at the LP stage compared to heterozygous controls (either $InR^{E19}/+$ or $InR^{GC25}/+$) and *OR*. Consistent with our hypothesis, we found that SGP cell number was significantly smaller in $InR^{E19/GC25}$ compared to *OR* (Figure 2.15B). In heterozygosis both *InR* alleles had significantly reduced SGP cell number (Figure 2.15B) and TF cell size (Figure 2.17B), but TF cell number (Figure 2.16B) and TF

number (Figure 2.15A) were not significantly different from *OR*. TF cell size was also reduced in $InR^{E19/GC25}$ trans-heterozygotes compared with controls and *OR* (Figure 2.17B), but as observed for lineage-specific cell size differences (Figure 2.5B), this did not account for the reduction in TF number (Figure 2.15A). Swarm cell migration was not affected, so that the anterior/posterior proportions of somatic cells was similar between $InR^{E19/GC25}$, controls, *OR* and *Ds*, but significantly different compared to *Ind* and *bab:GAL4>>*$bab2^{RNAi}$ (Figures 2.14, 2.15C). We did observe that in $InR^{E19/GC25}$ the proportion of anterior cells that differentiated into TF cells was elevated compared to controls and all three wild type lineages (Figure 2.15C). TF cell number per TF stack was also increased relative to controls ($p<0.001$; Figure 2.17C). Consistent with recent reports on a role for hormonal signaling in germ line stem cell niche formation (Gancz et al., 2011), these observations suggest that in addition to controlling cell proliferation, insulin signaling may also play a role in TF cell fate specification and morphogenesis. Nevertheless, these changes in TF cell allocation and morphogenesis do not compensate for the reduced number of anterior somatic cells in $InR^{E19/GC25}$ ovaries (Figure 2.16A), so that the ultimate result is specification of fewer TF cells and fewer TFs (Figure 2.15A, Figure 2.16B). Therefore, reduced insulin signaling lowers ovariole number principally through reducing the number of SGP cells established during embryogenesis, rather than through changes in larval ovarian development. In this way, reduced insulin signaling phenocopies the essential developmental differences that cause ovariole number difference between *OR* and *Ds*.

Discussion

Convergent evolution of reduced ovariole number by distinct developmental mechanisms

We have shown that independent instances of evolutionary reduction in ovariole number can result from alterations in different developmental processes (Figure 7). In *Ds*, a smaller somatic gonad primordium than that of *OR* is established by hatching, although L0 germ cell number and all other later ovariole developmental processes that we examined are similar between the two species. *Ds* therefore has fewer of all somatic cell types of the ovary, including TF cells, and as a consequence forms fewer TFs and fewer ovarioles. In contrast, the L0 gonad of *Ind* is initially the same size as that of *OR*. During larval development, a smaller proportion of the somatic gonad cells in *Ind* are allocated to TF fate due to differences in somatic cell migration within the gonad. As a result, fewer TFs and fewer ovarioles are formed.

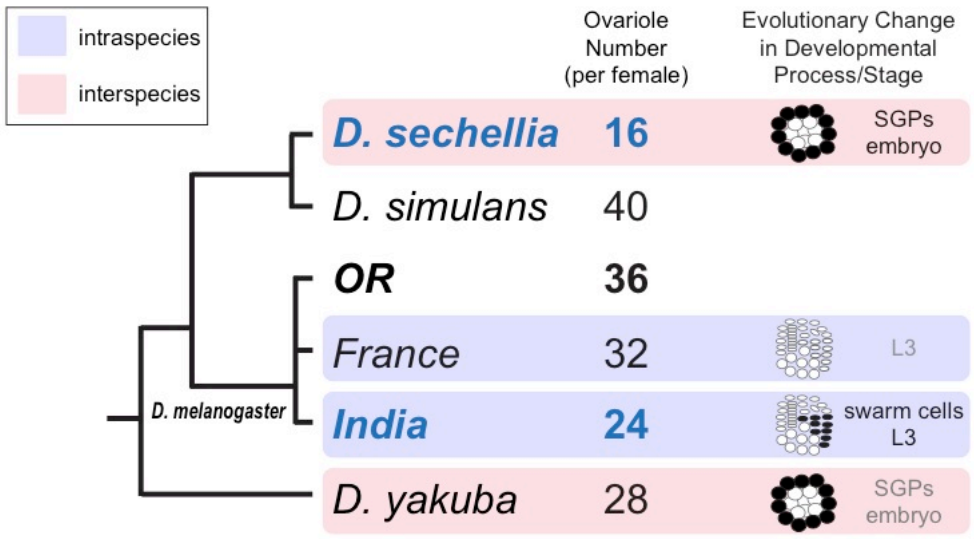


Figure 2.18: Different developmental mechanisms underlie ovariole number evolution. Lineages studied in detail in this report are shown in bold. Independent reductions in ovariole number (blue text) evolved in the *melanogaster* subgroup via distinct developmental mechanisms that affect different cell types and developmental stages.

Different genetic mechanisms independently regulate these different developmental processes in *D. melanogaster*. Reduction of insulin signaling pathway activity results in fewer L0 gonad primordium cells and fewer TFs, but does not affect migration behaviors later in ovarian development. In contrast, loss of *bab2* function in somatic gonad cells alters their migration behaviors, but does not affect L0 gonad size. Taken together with QTL linkage of *InR* and *bab* to ovariole number variation, this suggests that changes in distinct genetic pathways may underlie modular evolution of ovariole number in *Drosophila*, which could contribute to the high evolutionary lability of this trait.

We also note an interesting agreement between the developmental differences we observe between *OR, Ind*, and *Ds*, their candidate genetic bases, and previous inter- and intraspecies QTL analyses suggesting that the genetic basis for change in this trait may be different between and within *Drosophila* species (Bergland et al., 2008; Orgogozo et al., 2006). We have found that different developmental processes produce the TF cell number differences underlying ovariole number differences in *D. yakuba* (Sarikaya et al., 2012) and in the *D. melanogaster France* strain, both of which have fewer ovarioles than *OR*. The size of the L0 ovarian primordium is similar to *OR* in *France*, but significantly smaller than *OR* in *D. yakuba* (Figure 2.19). Thus for *France* and *D. yakuba*, as for *Ind* and *Ds*, ovarian primordium size differences are at the root of ovariole number variation between species, but within species larval developmental processes are the source of variation in this trait.

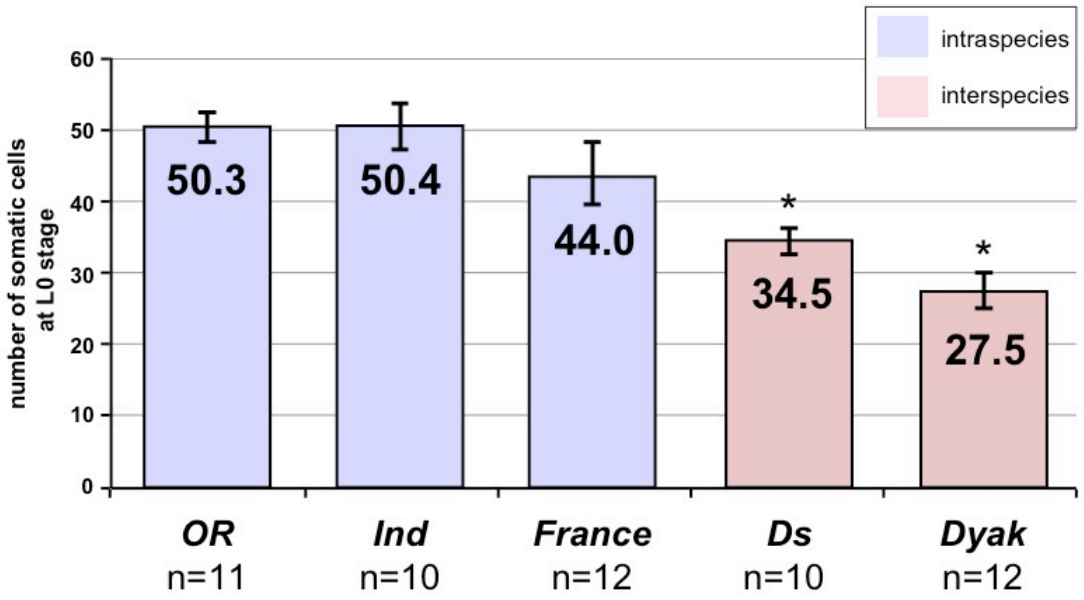


Figure 2.19: Inter- and intra-species comparisons of SGP cell number. Mean somatic ovarian cell number at first larval instar (L0) 0-3 hours after hatching (h AH) in the *Ind*, *OR* and *Ds* lineages discussed in the main text, as well as the *D. melanogaster* strain *France* and the species *D. yakuba* (*Dyak*), both of which have significantly lower average ovariole numbers than *OR*. Different *D. melanogaster* strains are blue bars; different *Drosophila* species (*D. sechellia* and *D. yakuba*) are pink bars. *D. yakuba* and *D. sechellia* L0 somatic gonad cell numbers are both significantly smaller (* $p<0.01$) than all three *D. melanogaster* strains, corresponding to their lower ovariole number. In contrast, *D. melanogaster* strains are not significantly different from each other, despite the lower ovariole number of *Ind* and *Fra* compared to *OR* (Figure 2.18).

Multiple developmental mechanisms affecting ovariole number may provide different opportunities for evolutionary change

Because ovariole number is determined by TF number at the larval-pupal transition, it is a complex trait that requires multiple developmental processes: embryonic establishment of the somatic gonad, proliferation during larval life, migration of a specific proportion of somatic gonad cells, differentiation of some anterior somatic cells into TF cells, and finally TF cell stacking to form TFs (Figure 2.3). Each of these steps is directed by very different mechanisms, each of which could conceivably be the target of evolutionary change. We propose that evolutionary change in ovariole number may be particularly likely to proceed via a diverse set of developmental and possibly genetic mechanisms because of its cell type complexity. The ovary is composed of multiple cell types that each follows an individual developmental program, and yet must be integrated to form a functional organ. The *Drosophila* sex combs are a similar example of a complex multicellular structure whose convergent evolution can proceed through multiple different developmental mechanisms {Kopp:2011bv}. Interestingly, both ovariole number and sex comb morphology show high evolutionary lability, perhaps indicating that complex traits provide a broader "evolutionary change landscape" that allows for rapid diversification.

In many cases where convergent morphological traits evolve via changes in the same genetic mechanisms, these morphological traits are terminal differentiation aspects of a single type of somatic cells. For example, the degree of expression of a pigment synthesis pathway (Protas et al., 2006), or the accumulation of cortical actin that determines the formation of an epidermal bristle (Sucena et al., 2003), are likely to be

processes that are cell-autonomous and do not require significant coordination with other cell types. The developmental processes operating prior to this differentiation will surely require cooperation of multiple cell types, but a single cell expresses pigment or develops a bristle autonomously. We speculate that this developmental feature may facilitate convergent evolution of these phenotypes. In contrast, ovarioles are multicellular rather than cell-autonomous structures, and as such evolution may have many "opportunities" to change this and other complex traits through multiple genes directing several distinct processes at different times in development.

Towards the genetic basis of ovariole number variation

Our functional experiments in *D. melanogaster* revealed two different genes that can regulate ovariole number in different ways. Loss of function of *InR* and *bab* not only reduce ovariole number in *D. melanogaster*, but do so by affecting the same developmental mechanisms that reduce ovariole number in *D. sechellia* and *D. melanogaster Ind*, respectively. The data shown here provide, to our knowledge, the first functional test of specific candidate genes within QTL linked to ovariole number variation (Bergland et al., 2008; Orgogozo et al., 2006; Wayne et al., 2001), and suggest these genes as potential causal loci for change in this trait. We speculate that evolution at the *bab* locus may underlie reduced ovariole number in *Ind*, while changes of *InR* function could be responsible for the evolution of reduced ovariole number in *D. sechellia*. Evolutionary changes at the *bab* locus have been previously implicated in the evolution of adult abdominal pigmentation and trichome patterns in *Drosophila* species (Gompel and Carroll, 2003; Kopp et al., 2000), but the nature of the selective pressures

acting on this locus are poorly understood. *bab* plays multiple roles in development, including TF formation (Godt and Laski, 1995; Sahut-Barnola et al., 1995), leg development (Couderc et al., 2002), and a role in somatic ovary cell migration that we describe here for the first time. The role of *bab* in ovariole number is likely to have a direct impact on fertility and therefore fitness. It may be that ectodermal patterning variation resulting from *bab* modification is a secondary effect of selection on *bab's* role in ovarian morphogenesis, or vice versa.

With respect to *InR*, several lines of evidence suggest that evolutionary change in insulin signaling (INS) genes plays an important role in ovariole number variation. First, consistent with its QTL linkage to interspecies ovariole number variation (Orgogozo et al., 2006), the specific developmental processes affected by *InR* loss of function correspond to those that vary between *Drosophila* species. Second, clinal variation in *InR* alleles has been observed in natural populations of *D. melanogaster* (Paaby et al., 2010), and ovariole number also exhibits clinal variation (Boulétreau-Merle et al., 1992; Collinge et al., 2006; David and Bocquet, 1975; Delpuech et al., 1995; Gibert et al., 2004; Paaby et al., 2010; Schmidt et al., 2005; Wayne et al., 2005). Third, analysis of clinal alleles reveals evidence of positive selection at the *InR* locus (Paaby et al., 2010). Finally, different organs are known to respond differently to changes in INS in *Drosophila* (Shingleton et al., 2005; Tang et al., 2011), providing mechanisms for putative organ-specific responses to changes in a global hormonal pathway, and consistent with the altered correlations between ovariole number and overall body size between species (Bergland et al., 2008; Hodin and Riddiford, 2000, this report).

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Chapter Three

Insulin Signaling Underlies Both Plasticity and Divergence of a Reproductive Trait in *Drosophila*

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Abstract

Phenotypic plasticity is the ability of a single genotype to yield distinct phenotypes in different environments. The molecular mechanisms linking phenotypic plasticity to the evolution of heritable diversification, however, are largely unknown. Here we show that insulin/insulin-like growth factor signaling (IIS) underlies both phenotypic plasticity and evolutionary diversification of ovariole number, a quantitative reproductive trait, in *Drosophila*. IIS activity levels and sensitivity have diverged between species, leading to both species-specific ovariole number and species-specific nutritional plasticity in ovariole number. Plastic range of ovariole number correlates with ecological niche, suggesting that the degree of nutritional plasticity may be an adaptive trait. This demonstrates that a plastic response conserved across animals can underlie the evolution of morphological diversity, underscoring the potential pervasiveness of plasticity as an evolutionary mechanism.

Introduction

Phenotypic plasticity is the ability of a single genotype to yield distinct phenotypes in different environments. Phenotypic plasticity may play an important role in evolutionary diversification, as it is capable of generating striking examples of biodiversity, including differences in morphology, behavior, life history, and species interactions (Moczek et al., 2011). However, whether or not phenotypic plasticity promotes or impedes evolutionary diversification is still unclear, and has been under debate for decades (West-Eberhard, 2003). One hypothesis is that common molecular mechanisms underlie both plasticity and interspecific variation in a trait, which would allow plasticity to promote diversification by providing a range of phenotypes whose

underlying genetic variation can be subject to selection by genetic accommodation, genetic assimilation, or other means (Waddington, 1942). The molecular underpinnings of plasticity within a single species are known for several systems (Abouheif and Wray, 2002; Brakefield et al., 1996; Rutherford and Lindquist, 1998; Suzuki, 2006), and there is also evidence that plasticity contributes to species differentiation (Bloom et al., 2013). However, specific examples that functionally demonstrate the molecular basis of both the plasticity and interspecies divergence of the same trait are lacking. We therefore sought to provide such an example, by examining the molecular basis of the evolutionary divergence and of the phenotypic plasticity of a single trait.

Reproductive traits are particularly relevant models for investigating the molecular mechanisms of phenotypic plasticity and evolutionary changes, because they affect the number of offspring, and hence fitness. Here we examine one such trait: insect ovariole number. Ovarioles are egg-producing structures of insect ovaries. At the anterior end of each ovariole is the germarium, where germ line stem cells (GSCs), supported within their somatic niche, self-renew and also differentiate to ultimately yield the mature oocyte and supporting germ cells. Posterior to the germarium, progressively maturing oocytes are arranged in an anterior to posterior progression within each ovariole.

Ovariole number spans three orders of magnitude across insects (Büning, 1994). Several lines of evidence suggest that ovariole number is adaptive. First, ovariole number is a strong determinant of reproductive capacity, and thus is positively correlated to female fecundity and fitness (Boulétreau-Merle et al., 1982; Cohet and David, 1978; David, 1970; Klepsatel et al., 2013). Second, ovariole number is heritable and lineage-specific. Quantitative and developmental genetic analyses suggest that inter- and intra-

species variation in ovariole number are controlled through multiple loci (Bergland et al., 2008; Orgogozo, 2006; Telonis-Scott et al., 2005; Wayne et al., 2001; 1997; Wayne and Mackay, 1998; Wayne and McIntyre, 2002). Third, ovariole number shows latitudinal and altitudinal clinal variation on multiple continents (Collinge et al., 2006; Wayne et al., 2005). In two cosmopolitan *Drosophila* species, *D. melanogaster* and *D. simulans*, ovariole number is greater in temperate populations than in tropical populations (Capy et al., 1994). Finally, ovariole number is correlated with species ecology. Low ovariole numbers commonly evolve among ecological specialists, whereas generalists, or insects with more heterogeneous food sources, tend to evolve higher ovariole numbers (Fitt, 1990; Kambysellis and Heed, 1971; Leather et al., 1988; Montague et al., 1981).

Ovariole number exhibits strong phenotypic plasticity in response to larval rearing environment, particularly nutrition (Hodin and Riddiford, 2000; Tu and Tatar, 2003) and temperature (Delpuech et al., 1995). Previous attempts to relate genetically fixed variation in and phenotypic plasticity of ovariole number in *Drosophila* concluded that different developmental mechanisms were responsible for species-specific ovariole number and ovariole number plasticity (Hodin and Riddiford, 2000). However, the underlying molecular mechanisms remained unknown. Many developmental genetic details underlying ovariole number determination have since emerged (Bartoletti et al., 2012; Green and Extavour, 2012; Sarikaya et al., 2012), allowing for molecular investigations of the basis of ovariole number determination and divergence. In the following section, we describe the essential cellular behaviors involved in ovariole morphogenesis. These developmental events suggest specific candidate processes and molecular mechanisms that may underlie the evolution of variation in ovariole number.

Ovary morphogenesis in *Drosophila* begins with the specification of somatic gonad precursor cells in late embryogenesis (Boyle and DiNardo, 1995). Unlike most larval tissues in *Drosophila*, somatic ovarian cells proliferate continuously throughout larval life with no dramatic cell death (King, 1970). Ovariole morphogenesis begins with the stacking of somatic ovarian cells into structures called terminal filaments (TFs) in the anterior of the larval ovary (Godt and Laski, 1995). TF number at the larval-pupal transition (LP) stage directly determines adult ovariole number (Hodin and Riddiford, 2000), and the number and morphogenesis of TF cells at LP stage determines TF number (Sarikaya et al., 2012). Somatic ovarian cells are then specified as anterior versus posterior cells, and a constant percentage of the anterior cells become TF cells (Green and Extavour, 2012). Insulin and ecdysone signaling regulate TF cell number through modulating somatic ovarian cell proliferation, differentiation, and morphogenesis (Gancz et al., 2011; Green and Extavour, 2012; Hodin and Riddiford, 1998). This suggests that variation in hormonal signaling could underlie one or both of species-specific ovariole number and the phenotypic plasticity of ovariole number.

Here we examine the role of insulin/insulin-like growth factor signaling (IIS) in the determination of mean ovariole number and the phenotypic plasticity of ovariole number in *Drosophila*. Furthermore, we use a comparison of two *Drosophila* species, *D. melanogaster* and *D. sechellia*, to investigate the hypothesis that the same molecular mechanism regulates both species-specific values and phenotypic plasticity of the same trait, ovariole number.

Materials and Methods

Drosophila *strains, culture conditions, and diet manipulations*

The following strains were used as wild type strains for species comparisons: *D. melanogaster* OregonR-C (Bloomington Drosophila Stock Center (BDSC) #5; gift of the Hartl lab, Harvard University), *D. sechellia* Robertson strain (UC San Diego *Drosophila* Species Stock Center (DSSC) #14021-0248.25; gift of the Hartl lab), *D. simulans* (DSSC #14021-0251.194), and *D. erecta* (DSSC #14021-0224.01). To evaluate the amount of intraspecies variation in ovariole number, we counted adult ovariole number in isofemale lines of *D. melanogaster* and *D. sechellia*. Both tropical and temperate populations of *D. melanogaster* were considered. Tropical *D. melanogaster* isofemale lines, established from a population in Zambia, were a gift of the Flatt Lab (University of Lausanne). North American *D. melanogaster* isofemale lines (derived from females collected in Nevada, Catalina Island, CA, Santa Fe, NM, and Raleigh, NC) were a gift of the DePace lab (Harvard Medical School). *D. sechellia* isofemale lines were a gift of the Hartl lab.

To examine IIS function in *D. melanogaster*, the following lines were used: the InR^{339} hypomorphic allele (Brogiolo et al., 2001; Fernandez et al., 1995) a gift of the Hafen lab (ETH Zurich)); the InR^{GC25} inversion allele (BDSC #9554; (Chen et al., 1996)); and the *Df(3R)Exel6186* deficiency allele (BDSC #7647).

To determine the role of systemic IIS from brain-derived peptides, we genetically ablated the principle insulin-producing cells of the brain. We used the *dilp2-Gal4* driver (Wu et al., 2005), which is expressed specifically in the paired small clusters of medial neurosecretory cells that are known to produce Drosophila insulin-like peptides. We

crossed this driver to the *UAS-rpr* (BDSC #5824) line to drive expression of the proapoptotic gene *reaper*.

To determine the responsiveness of somatic ovarian cells to IIS, we altered expression of the *Drosophila* insulin-like receptor *InR* specifically in the ovary by using the *c587-GAL4* driver, which is expressed specifically in somatic ovarian cells beginning in the third larval instar ((Manseau et al., 1997); gift of the Drummond-Barbosa lab, Johns Hopkins University). We crossed this driver to the following *UAS* lines to alter *InR* activity: UAS-InR^{Exel} (BDSC #8262), *UAS-*InR^{K1409A} (BDSC #8259), and *UAS-*InR^{RNAi} (BDSC #31037).

All adult ovariole counts and larval-pupal transition (LP) stage TF counts were performed as previously described(Sarikaya et al., 2012). Tibia length (adult females) was used as an adult body size proxy, as it has been previously demonstrated to correlate positively with body mass, which is indicative of overall body size (Catchpole, 1997).

Flies were maintained on standard lab diet (32g Torula yeast, 60.5g corn meal, 128g dextrose, 9.2g agar per liter). In all diet manipulation experiments, flies were raised from egg through to adult on the specified diet. Rich diet for all analyses consisted of standard lab diet supplemented with active dry yeast. Poor diet consisted of standard lab diet diluted with 3% agar in a ratio of 1:3 (25% final concentration standard lab diet) with no dry yeast supplementation. Wortmannin (EMD Millipore) was dissolved in 100% methanol and added to standard lab diet at 1% v/v. All rearing and experiments were performed at 25°C at 60-70% humidity.

Quantitative PCR

As one measure of IIS pathway activity, we measured levels of *Thor* transcript (Puig, 2003). Total RNA was extracted from ten biological replicates of five whole wandering third instar females that were grown on rich diet. RNA was extracted using Trizol (Invitrogen), treated with TURBO DNase-I (Ambion, Life Technologies), and phenol-chloroform extracted. cDNA was prepared using oligo-dT primers and 0.5µg RNA per reaction with Superscript III First Strand Synthesis Kit (Invitrogen). qPCR was performed using PerfeCta SYBR Green SuperMix, Low Rox (Quanta Biosciences). *gapdh1* was used to normalize RNA levels and *rp49* was used an expression control. Primer pairs were designed for use with both species templates. Primers were verified by performing species-specific standard curves for each primer pair, and showed <2.5% difference in amplification efficiency between species. Primer pairs were as follows: *gapdh1*-f, AGCCGAGTATGTGGTGGAGT, *gapdh1*-r, GGCTGTAGGCGTCCAGGTTA; *Thor*-f, AGCTAAGATGTCCGCTTCACC, *Thor*-r, TTTGGTGCCTCCAGGAGTGG; *rp49*-f, TGCTAAGCTGTCGCACAAATG, *rp49*-r, TTCTTGAATCCGGTGGGCAG.

Immunohistochemistry, confocal imaging, and analysis

Immunostaining was carried out as previously described (Sarikaya et al., 2012). The following primary antibodies were used: mouse 4D9 anti-Engrailed (1:40, Developmental Studies Hybridoma Bank), rabbit anti-Vasa (1:500, gift of P. Lasko, McGill University), rabbit anti-phospho-*Drosophila* Akt (Ser505) (1:200, Cell Signaling Technology #4054). Secondary reagents used were Hoechst 33342 (Sigma, 1:1000 of 10

mg/ml stock solution), goat anti-mouse Alexa 488, goat anti-guinea pig Alexa 488, and donkey anti-rabbit Alexa 555 (1:500, Invitrogen). Samples were mounted in Vectashield (Vector labs) and imaged using a Zeiss LSM 780 confocal microscope.

Phosphorylated Akt (phospho-Akt) staining was quantified by measuring mean fluorescence signal intensity from maximum projection images composed of an equal number of confocal z-slices for each ovary. Secondary-only controls (Figure 3.1B, 3.1D) indicate that the staining detected (Figure 3.1A, 3.1C) and measured (Figure 3.4B) is not background signal. A standard area of specifically anterior somatic ovarian cells, the cells from which terminal filament precursor cells are specified, was analyzed. Phospho-Akt intensity was normalized to mean DNA (Hoechst 33342) staining intensity to control for potential differences due to specific immunostaining experiments. Images were analyzed with ImageJ 1.45I.

Statistical Analyses

Student's *t* test was used for all pairwise comparisons of differences in means unless otherwise noted. Bonferroni adjustment for multiple comparisons was performed as appropriate. Mann-Whitney (Wilcoxon) test was used to evaluate differences in phospho-Akt staining intensity. To evaluate differences in interpopulational variation in ovariole number, Bartlett's test was used, as this test does not assume homogeneity of the variance of species-specific variances. Homo-/heterogeneity of species-specific variances were tested with Welch ANOVA (Welch *t*) to account for differences in mean values. Correlations, where noted, were evaluated by least squares linear regression of mean values for each genotype. Statistical analyses were performed in Excel and JMP Pro 11.

Results

Role of systemic IIS in determining ovariole number.

We previously showed that loss of function of the *Drosophila* insulin-like receptor (*InR*) in *D. melanogaster* significantly reduces TF number by reducing both the number of somatic gonad precursor cells and the subsequent somatic cell proliferation rate throughout larval life (Green and Extavour, 2012). To determine if TF number reduction in *InR* mutants is due to autonomous IIS activity in somatic ovarian cells rather than through an indirect mechanism, we first asked whether IIS is active in ovarian cells at the relevant developmental time. Phosphorylated Akt (phospho-Akt) protein, an indicator of active IIS, was detectable at levels above background in wandering third larval instar ovaries, the time at which TF cells are proliferating and TFs are forming (Figure 3.1A-D; compare A with B, and C with D). Phospho-Akt was also detected at above-background levels in the fat body, however at lower levels than in the ovary (Figure 3.1A-D). We then used the somatic ovary-specific driver *c587-GAL4* to abrogate or increase IIS specifically in the ovary. When IIS was decreased in the ovary either with the dominant negative *InR* allele *K1409A*, or with an *InR* RNAi construct, ovariole number was significantly decreased ($p<0.01$ for InR^{K1409A}, $p<0.01$ and $p=0.08$ for *c587-GAL4* and $UAS{:}InR^{RNAi}$ parental controls, respectively) (Figure 3.2). Conversely, when IIS was increased with overexpression of wild type *InR* (InR^{Exel}), ovariole number was significantly increased ($p<0.01$) (Figure 3.2). As expected due to the use of an ovary-specific GAL4 driver, these changes in ovariole number were not simple consequences of changes in body size (Table 3.1). Finally, to determine if systemic IIS from brain-derived insulin-like peptides (dILPs) regulates

Table 3.1: Body size for ovary-specific genetic manipulations of INS in *D. melanogaster*.

Genotype	Body size, µm (*n*)	95%CI	*p*
c587-GAL4/+; CyO P{Act:GFP}JMR1 / +	523.6 (20)	6.1	0.002
c587>InR^{K1409A}	538.6 (20)	6.2	
c587-GAL4	510.3 (24)	8.0	0.02
UAS:InR^{RNAi}	501.6 (31)	5.6	1E-5
c587>InR^{RNAi}	522.1 (20)	5.8	
c587-GAL4/+; CyO P{Act:GFP}JMR1 / +	520.7 (22)	7.0	0.14
c587>InR^{Exel}	527.4 (30)	5.3	

Table 3.1: Body size for ovary-specific genetic manipulations of INS in *D. melanogaster*. Tibia length of adult females was used as an adult body size proxy (Catchpole, 1997). Shading indicates groups of experimental flies and their corresponding controls. *p*-values (Student's *t*-test) in each group refer to differences between ovariole numbers in females carrying both the c587-*GAL4* driver and the indicated *UAS-InR* construct and in control females. Controls were siblings carrying a balancer chromosome rather than the UAS construct in the case of *InR^{K1409A}* and *InR^{Exel}*, or individual parental genotypes in the case of *InR^{RNAi}*, which is a homozygous viable construct. Bonferroni adjustment for multiple hypothesis testing was performed to compare *c587>InR^{RNAi}* to two parental controls ($p<0.025$ new significance threshold). There is no correlation between ovariole number and body size in ovary-specific manipulations (see Figure 3.2; Pearson's correlation coefficient $R=0.44$, $p=0.32$). This is consistent with our previous finding (Green and Extavour, 2012; Sarikaya et al., 2012) that proliferation of ovarian somatic cells, specifically, determines ovariole number.

ovariole number determination, we genetically ablated insulin-producing neurons by using a *dilp2-GAL4* driver to overexpress the proapoptotic gene *reaper* (*rpr*). Adult ovariole number was significantly reduced in *dilp2>rpr* females compared to *UAS:rpr* control females (Figure 3.3; $p<0.001$). Taken together, these results show that systemic IIS from brain-derived dILPs controls autonomous somatic ovarian cell proliferation, and modulation of IIS leads to changes in ovariole number.

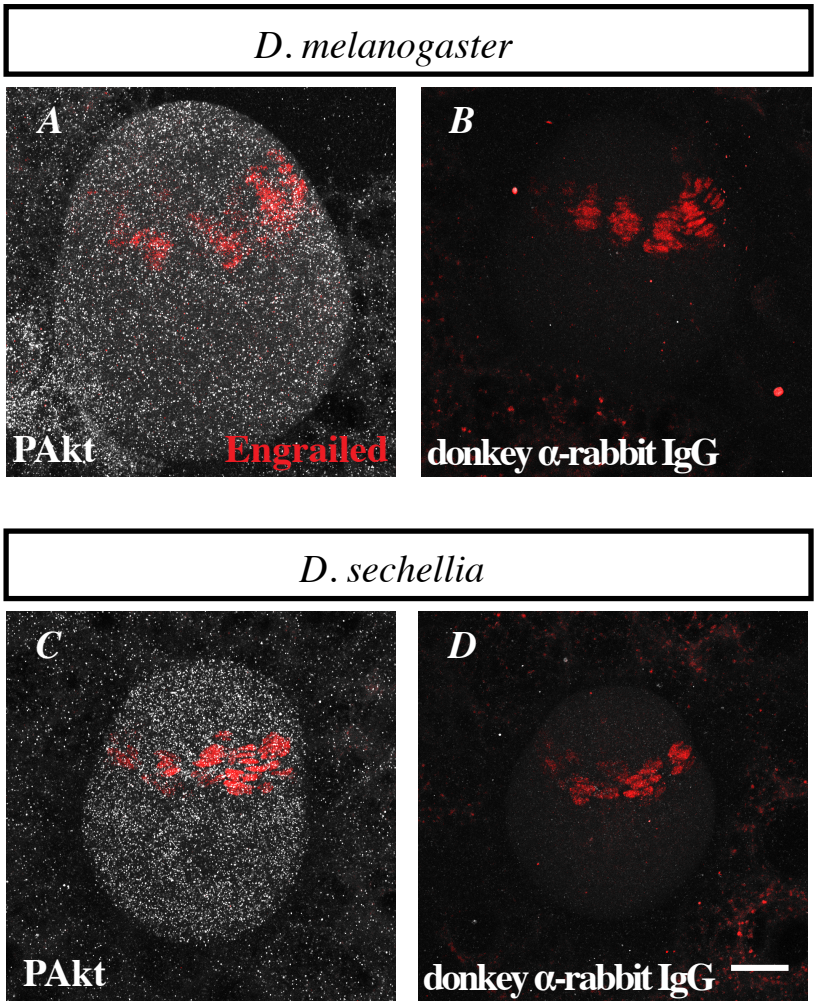


Figure 3.1: Insulin signaling is active in larval ovaries of *Drosophila*. (*A-D*) IIS activity in larval ovaries of both *D. melanogaster* and *D. sechellia* visualized by phosphorylated Akt (pAkt: white) (maximum projection of optical sections through whole ovary). *Engrailed* (red) marks terminal filament precursor cells. (*B*), (*D*) Secondary antibody-only controls. Scale bar = 20µm.

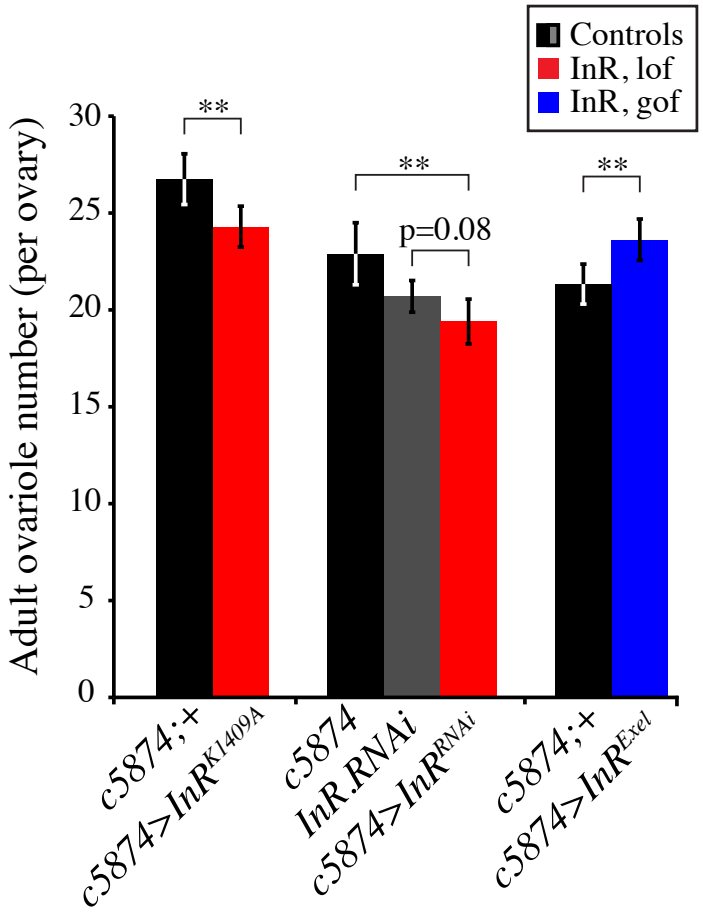


Figure 3.2: Insulin signaling regulates ovariole number determination in *Drosophila*. Adult ovariole number in females with ovary-specific expression of *InR* alleles driven by the c587-GAL4 driver. $n \geq 20$ ovaries for all genotypes. For InR^{K1409A} and InR^{Exel}, controls are siblings carrying a balancer chromosome (black bars). $n \geq 20$ for all genotypes. Error bars show 95% CI of means. Student's *t*-test: ***$p<0.001$, **$p<0.01$.

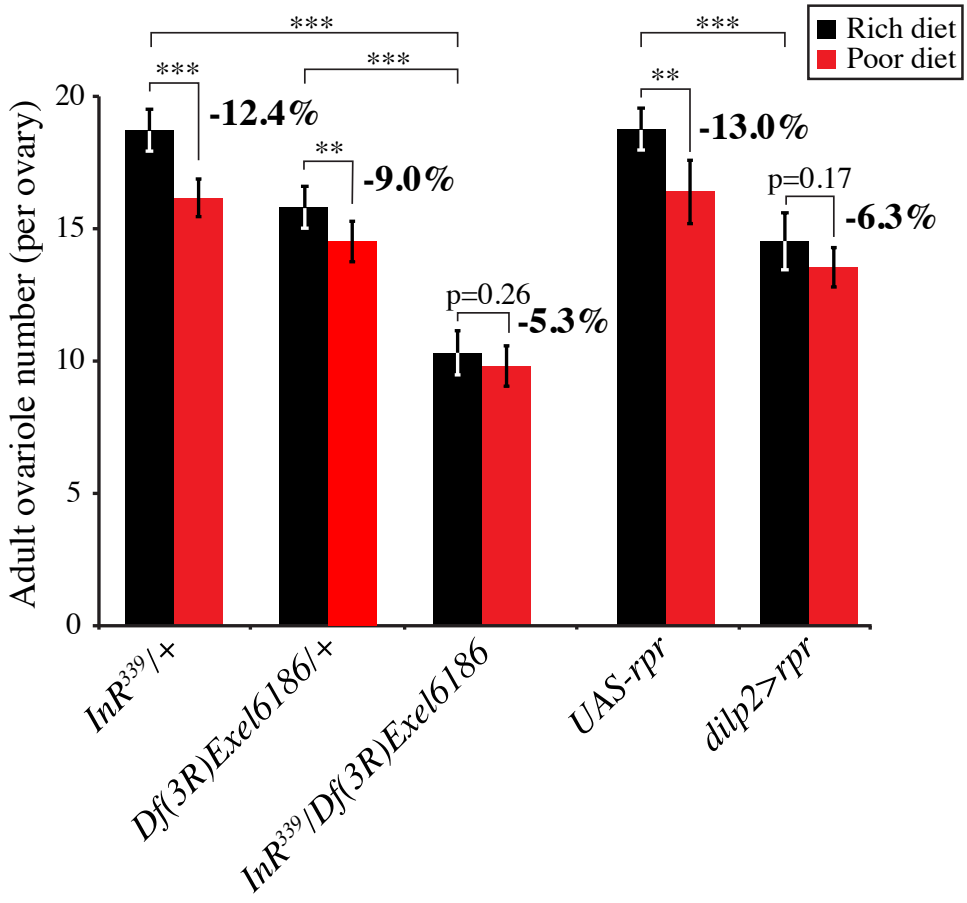


Figure 3.3: Insulin signaling regulates ovariole number plasticity in *Drosophila*. Adult ovariole number in females with loss of *InR* function (*InR^{339}/Df(3R)Exel6186*) or with Dilp-producing neurons ablated (*dilp2-GAL4>UAS:rpr*), reared on rich or poor diets. $n \geq 20$ for all genotypes. Error bars show 95% CI of means. Student's *t*-test: ***$p<0.001$, **$p<0.01$.

Role of IIS in nutritional plasticity of ovariole number.

Systemic IIS is nutritionally controlled (Hietakangas and Cohen, 2009). To test if IIS mediates the nutritional plasticity of ovariole number, we reared flies with wild type or modulated levels of IIS on rich or poor diets (see Methods). Like in wild type flies (Sarikaya et al., 2012), ovariole numbers were significantly reduced by poor diet in heterozygotes for *InR* loss of function mutations or *UAS:rpr* controls (Figure 3.3; $p<0.001$ and $p<0.01$, respectively). Body size of flies with altered IIS levels showed a more variable response to poor diet than wild type flies, and body size was not a reliable predictor of ovariole number across genotypes (Table 3.2). However, *InR* loss of function mutant and *dilp2>rpr* females showed no statistically significant change in ovariole number on rich versus poor diet (Figure 3.3; $p=0.39$ and $p=0.17$, respectively). These results show that IIS is a molecular mediator of nutritional plasticity of ovariole number in *D. melanogaster*.

Table 3.2: Body size for genetic manipulations of INS under different nutritional conditions in *D. melanogaster*.

Genotype	Diet	Body size, µm (*n*)	95%CI	*p*	% Change
InR^{339}/+	Rich	478.0 (20)	6.1	3.3E-5	
	Poor	457.1 (20)	5.0		-4.4
Df(3R)Exel6186/+	Rich	483.5 (20)	6.6	0.04	
	Poor	473.9 (20)	6.3		-2.0
InR^{339} / Df(3R)Exel6186	Rich	356.2 (34)	4.7	0.03	
	Poor	365.5 (26)	5.6		+2.6
UAS-rpr	Rich	504.0 (26)	10.3	0.09	
	Poor	515.5 (20)	7.6		+2.2
dilp2>rpr	Rich	452.1 (30)	3.8	9.4E-5	
	Poor	434.7 (20)	6.6		-3.8

Table 3.2: Body size for genetic manipulations of INS under different nutritional conditions in *D. melanogaster*. Tibia length of adult females was used as an adult body size proxy (Catchpole, 1997). Shading indicates groups of flies with the same genotype raised on rich or poor diet. *p*-values (Student's *t*-test) refer to difference between body size of flies of the same genotype raised on rich and poor diets. Bonferroni adjustment for multiple hypothesis testing yields $p<0.01$ as adjusted significance threshold. Percent change refers to body size reduction on poor diet compared to rich diet.

IIS activity and sensitivity in D. melanogaster *and* D. sechellia.

The *melanogaster* subgroup species *D. melanogaster* and *D. sechellia*, which diverged only five million years ago, have remarkably divergent mean ovariole numbers of 18.2 and 7.6, respectively. We previously showed that the heritable ovariole number difference between these species is caused by differences in somatic gonad precursor cell specification and somatic ovarian cell proliferation rate throughout larval life, and that *InR* loss of function mutants in *D. melanogaster* phenocopy both of these differences (Green and Extavour, 2012). We therefore hypothesized that IIS activity is reduced in *D. sechellia* compared to *D. melanogaster*. To test this hypothesis we measured transcript expression of the growth attenuator *Thor*, which is negatively regulated by IIS (Puig, 2003). 4E-BP, the protein product of the *Thor* transcript, is a known negative regulator of cell number in *Drosophila* (Puig, 2003). We found that *Thor* expression in *D. sechellia* was significantly greater than in *D. melanogaster* (Figure 3.4A; $p<0.001$). In addition, we quantified the levels of phospho-Akt in the larval ovary of both species, and found that these levels were significantly higher in ovaries of *D. melanogaster* than of *D. sechellia* (Figure 3.4B; $p<0.05$). Taken together, these assessments of IIS activity indicate that IIS operates at higher levels in *D. melanogaster* than in *D. sechellia*. Consistent with these results, body size of *D. sechellia* is significantly smaller than that of *D. melanogaster* (Table 3.3; $p<0.001$). This suggests that evolutionary changes in IIS contribute to the divergence in ovariole number between these two species.

Table 3.3: Body size for *D. melanogaster* and *D. sechellia* under Wortmannin treatment.

Genotype	[Wortmannin] µM	Body size, µm (*n*)	95%CI	*p*	% Change
D. melanogaster					
	0 (MeOH control)	490.9 (20)	5.5		
	0.5	481.3 (21)	7.6	0.05	-2.0
	1	490.8 (22)	7.5	0.98	0
	10	472.1 (18)	10.1	0.003	-3.8
D. sechellia					
	0 (MeOH control)	435.9 (18)	9.4		
	0.5	432.6 (22)	4.2	0.54	-0.8
	1	423.7 (23)	6.8	0.04	-2.8
	10	395.9 (6)	6.7	1.1E-6	-9.2

Table 3.3: Body size for *D. melanogaster* and *D. sechellia* under Wortmannin treatment. *D. sechellia* flies eclose at very low rates (<1%) at 10µM Wortmannin concentration. % change in body size from methanol control is shown for each species. *p*-values (Student's *t*-test) refer to difference between body size of flies grown on indicated Wortmannin treatment versus methanol control.

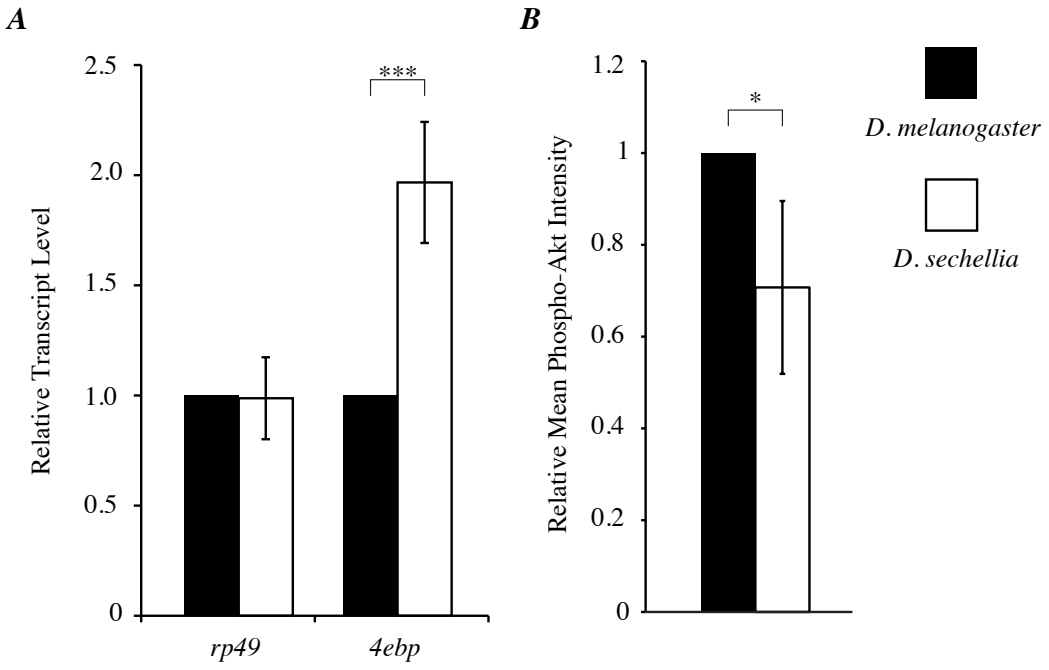


Figure 3.4: Differential IIS activity exists between *D. melanogaster* and *D. sechellia*. Quantified levels of (*A*) larval expression of the growth attenuator *Thor* (normalized to *gapdh1*) and the ribosomal protein gene *rp49* and (*B*) phospho-Akt intensity in wandering third instar larval ovaries of *D. melanogaster* compared to *D. sechellia*. $n \geq 10$ biological replicates (A) or ovaries (B) for all genotypes. Error bars show 95% CI of means. Student's *t*-test in (*A*); Mann-Whitney (Wilcoxon) test in (*B*): ***$p<0.001$, *$p<0.05$.

To further test for species-specific differences in IIS-mediated control of TF number between *D. melanogaster* and *D. sechellia*, we used interspecies hybrid complementation tests. Previous quantitative genetics analysis suggested that the *InR* locus may contribute to interspecies variation in ovariole number (Orgogozo, 2006). Therefore, we crossed *D. melanogaster* females carrying *InR* loss of function mutations with *D. sechellia* males, and counted TF number in resulting *melanogaster/sechellia* hybrids. Hybrids carrying mutant *InR* alleles from *D. melanogaster* had significantly reduced body size (Table 3.4) and TF number compared to control hybrids carrying a wild type *D. melanogaster InR* allele (Figure 3.5; $p<0.05$ for InR^{GC25} and *Df(3R)Exel6186*; $p<0.01$ for InR^{339}). This suggests that the wild type *D. melanogaster InR* allele may confer a higher level of IIS than the *D. sechellia* allele.

Table 3.4: Body size for *D. melanogaster*/*D. sechellia* hybrids.

Genotype	Body size, µm (*n*)	95%CI	*p*
Dmel Oregon R / Dsec	3104 (33)	48	
Dmel FRT82 InR^{339} / Dsec	3133 (38)	73	1E-5
Dmel TM3 P{w^{+mC}=Act:GFP}JMR2 Ser^{1} / Dsec	3425 (33)	94	
Df(3R)Exel6186 / Dsec	2965 (20)	37	9E-10
Dmel TM3 P{w^{+mC}=Act:GFP}JMR2 Ser^{1} / Dsec	3218 (31)	54	
Dmel In(3R)GC25, InR^{93Dj4} / Dsec	3064 (24)	49	2E-8
Dmel TM3 P{w^{+mC}=Act:GFP}JMR2 Ser^{1} / Dsec	3278 (29)	36	

Table 3.4: Body size for *D. melanogaster*/*D. sechellia* hybrids. Pupal length was used as a body size proxy for *D. melanogaster/D. sechellia* species hybrids because most hybrid genotypes fail to eclose, precluding measurement of tibia length. All pupae were female, as male hybrids die before wandering third larval instar. *p*-values (Student's *t*-test) refer to difference between hybrid carrying a *D. melanogaster InR* mutant allele and its corresponding sister control (comparison pairs indicated by shading), which carries a wild type *D. melanogaster InR* allele.

Because *D. sechellia InR* mutants are not available, we could not test this hypothesis directly by creating hybrids carrying a loss of function *D. sechellia InR* allele and a wild type *D. melanogaster InR* allele. However, if our interpretation is correct, then the decrease in ovariole number caused by loss of one functional *D. melanogaster InR* allele in should be less severe in *D. melanogaster* heterozygotes than in *melanogaster/sechellia* hybrids. Consistent with our hypothesis, adult ovariole number in *D. melanogaster InR* loss of function heterozygotes was not significantly different from wild type (*Oregon R*) for two different *InR* alleles, InR^{GC25} and InR^{339} (Figure 3.5; p=0.20, 0.34, respectively). For a third *D. melanogaster InR* loss of function allele, *Df(3R)Exel6186*, adult ovariole number was significantly lower than wild type (Figure 3.5; $p<0.001$), but the degree of reduction in ovariole was somewhat lower than that seen in the *melanogaster/sechellia* hybrid for the same *InR* allele (12.5% versus 13.6% reduction in ovariole number; Figures 3.3, 3.5). In summary, with these experiments we have compared the decrease in ovariole number caused by heterozygosis for a loss of function *D. melanogaster InR* allele in *D. melanogaster* heterozygotes versus *D. melanogaster/D. sechellia* hybrids, and shown that the ovariole number decrease is higher in the interspecies hybrids (Figure 3.5). Overall, these results are consistent with our hypothesis that the wild type *D. melanogaster InR* allele confers a higher level of IIS than the wild type *D. sechellia* allele, consistent with IIS activity being higher in *D. melanogaster* compared to *D. sechellia*. Taken together, these data demonstrate that IIS activity differs between *D. melanogaster* and *D. sechellia*, and that this activity difference contributes to species-specific ovariole number.

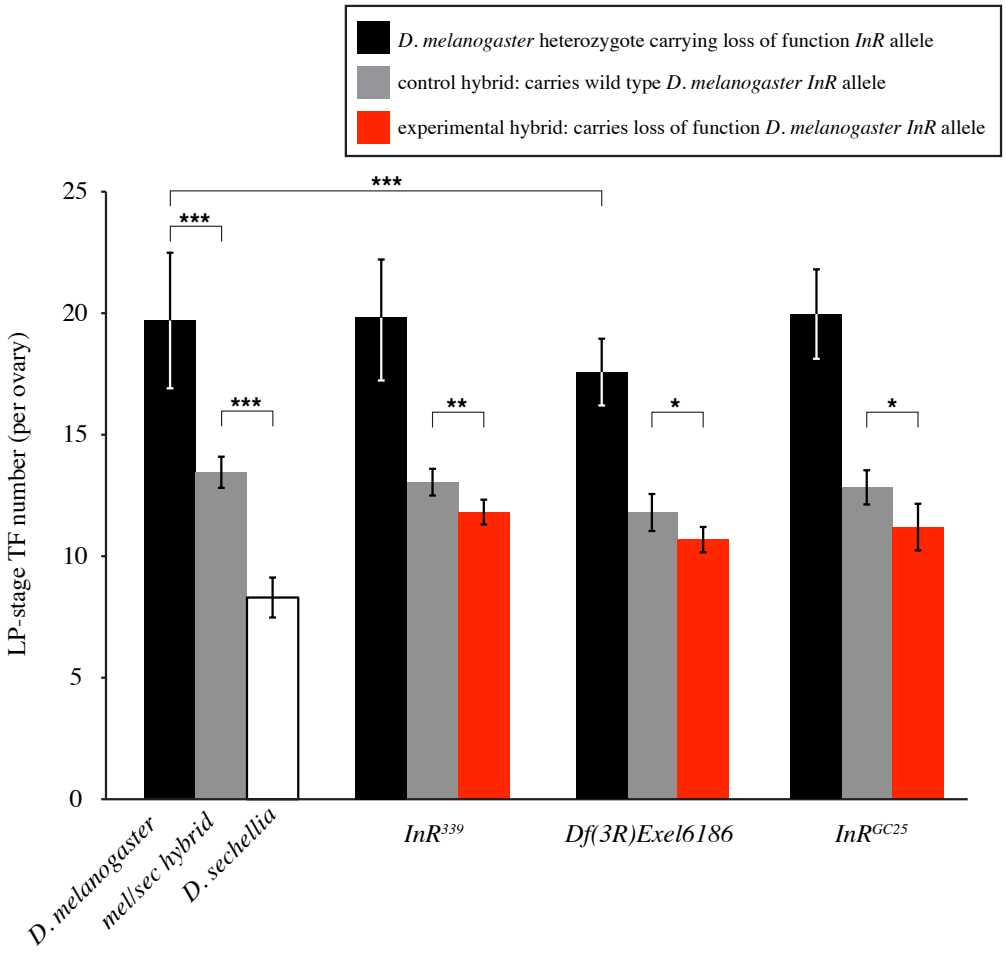


Figure 3.5: Interspecies hybrid complementation genetics suggests differential IIS activity between *D. melanogaster* and *D. sechellia*. Left-most set of bars shows TF number in *D. melanogaster* (black bar), *D. melanogaster/D. sechellia* hybrid (grey bar), and *D. sechellia* (white bar) females. Remaining sets show adult ovariole number in *D. melanogaster* females heterozygous for *InR* loss of function mutation (black bars), and final TF number in *D. melanogaster* InR^{*}*/D. sechellia* hybrids (where *InR** is any of three different *InR* loss of function alleles; red bars) compared to *D. melanogaster* InR^{+}*/D. sechellia* control hybrids (grey bars). Controls are sisters carrying a wild type copy of *D. melanogaster InR*. $n \geq 10$ for all genotypes. Error bars show 95% CI of means. Student's *t*-test: ***$p<0.001$, **$p<0.005$, *$p<0.05$.

IIS sensitivity controls differential plastic response to nutrition in several insect species (Emlen et al., 2012; Snell-Rood and Moczek, 2012; Tang et al., 2011). To determine how IIS activity difference could influence nutrition-dependent plasticity of ovariole number, we fed flies food containing Wortmannin, a specific inhibitor of PI3K (Yano et al., 1993), in a graded concentration series. Consistent with the results of genetic manipulation of IIS (Figures 3.2, 3.3, 3.5), body size (Table 3.3) and ovariole number (Figure 3*a*) were reduced in a dose-dependent manner in both species when grown on food containing Wortmannin. However, at all tested concentrations of Wortmannin, ovariole number was more significantly reduced in *D. melanogaster* than in *D. sechellia* (Figure 3.6A). This indicates that ovariole number is more sensitive to changes in IIS in *D. melanogaster* than in *D. sechellia*. Together with our finding of evolved differences in IIS between the two species, this also shows that that higher IIS activity in *D. melanogaster* is correlated with higher sensitivity to changes in IIS compared to *D. sechellia*.

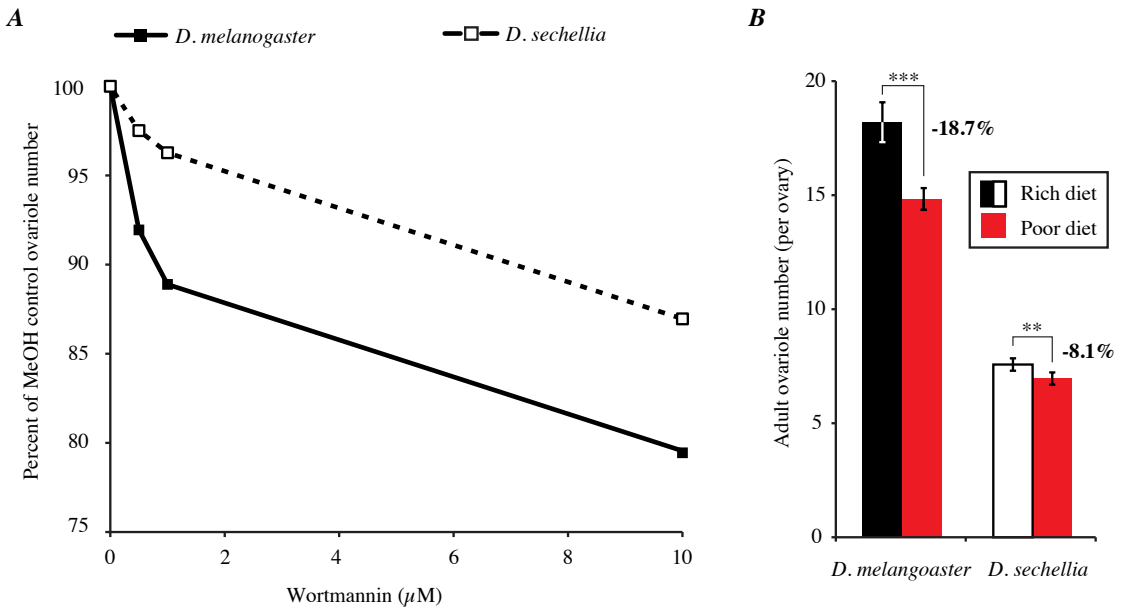


Figure 3.6: Differential IIS sensitivity between *D. melanogaster* and *D. sechellia* correlates with species-specific nutritional plasticity of ovariole number. (*A*) Dose-dependent Wortmannin-induced decrease in adult ovariole number in *D. melanogaster* and *D. sechellia*, shown as percent decrease relative to flies reared on control food containing methanol (Wortmannin solvent). n=20 for each species at all concentrations except *D. sechellia* at 10μM, n=6 (due to low eclosion rate of *D. sechellia* at high Wortmannin concentrations). (*B*) Adult ovariole number Poor diet in *D. melanogaster* and *D. sechellia* reared on poor and rich diets. Error bars show 95% CI of means. $n \geq 20$ for all genotypes and conditions. Student's t-test: ***$p<0.001$, **$p<0.005$.

Correlation between IIS sensitivity and nutritional plasticity.

To test if evolved differences in IIS activity levels and sensitivity could yield differences in nutritional plasticity between species, we measured ovariole number nutritional plasticity for *D. sechellia*. As in *D. melanogaster* (Sarikaya et al., 2012), poor diet reduced ovariole number in *D. sechellia*, but only by 8.1%, in contrast to 18.7% in *D. melanogaster* (Figure 3.6B). Body size was significantly reduced by poor diet in *D. melanogaster* ($p<0.001$), whereas in *D. sechellia* body size was reduced numerically but not significantly, ($p=0.08$) (Table 3.5). These data demonstrate that evolutionary change in IIS underlies the divergence of both mean ovariole number and the nutritional plasticity of ovariole number between these two *Drosophila* species.

Table 3.5: Body size for *Drosophila* species on rich versus poor diet.

Genotype	Diet	Body size, µm (*n*)	95%CI	*p*	% Change
D. melanogaster	Rich	505.4 (22)	6.4	1.1E-4	
	Poor	479.0 (38)	10.6		-5.2
D. sechellia	Rich	459.6 (17)	5.6	0.08	
	Poor	453.5 (31)	3.5		-1.3
D. erecta	Rich	432.3 (20)	5.3	0.22	
	Poor	426.9 (23)	6.8		-1.2
D. simulans	Rich	456.5 (25)	7.6	5.8E-7	
	Poor	429.1 (25)	5.0		-6.0

Table 3.5: Body size for *Drosophila* species on rich versus poor diet. Shading indicates groups of flies of the same species raised on rich or poor diet. Significance of difference between body size of flies raised on rich and poor diets assessed using Student's *t*-test. Percent change refers to body size reduction on poor diet compared to rich diet. There is no correlation between ovariole number and body size (see Figure 3.9A; Pearson's correlation coefficient R=0.13, p=0.76).

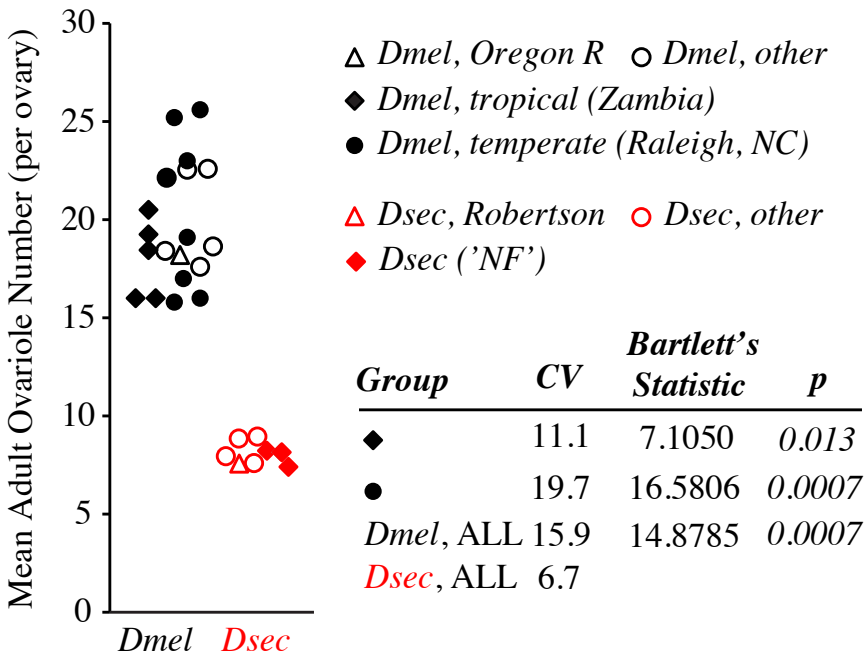
Interpopulational variation in ovariole number

If plasticity promotes diversification by providing a range of phenotypes whose underlying genetic variation can be subject to selection, then modulating the degree of plasticity may lead to differences in interpopulational divergence. Having observed that the degree of nutritional plasticity in ovariole number has diverged between *D. melanogaster* and *D. sechellia*, we asked if interpopulational variation in ovariole number also differs between these species. We measured mean ovariole number for multiple isofemale lines from both species, and observed greater between-population variation for ovariole number in *D. melanogaster* compared to *D. sechellia* (Figure 3.7, Table 3.6; $p<0.001$). Although *D. sechellia* occupies an exclusively tropical habitat while *D. melanogaster* is distributed worldwide (Figure 3.8), even when considering variation within a tropical *D. melanogaster* population, variation is significantly greater in *D. melanogaster* compared to *D. sechellia* (Figure 3.7, $p<0.05$). Genetic variation in *D. sechellia* is known to be lower than that of other *melanogaster* group species (Legrand et al., 2009), and it is possible that this contributes to its reduced interpopulational variation in ovariole number. However, we argue that IIS-dependent plasticity provides a proximate molecular mechanism for the evolutionary divergence of ovariole number. Our data are consistent with the idea that plasticity plays a central role in diversifying ovariole number not only between species, but also within species.

Table 3.6: Mean adult ovariole number for *D. melanogaster* and *D. sechellia* populations.

Genotype	Mean ON (*n*)	95%CI	Variance ON
D. melanogaster			
Oregon R-C	18.2 (40)	0.87	7.96
RAL 786	15.8 (10)	1.33	4.62
RAL 324	25.6 (10)	2.07	11.16
RAL 399	19.1 (10)	1.45	5.43
RAL 380	25.2 (10)	1.00	2.62
RAL 303	23.0 (10)	0.92	2.22
RAL 21	17.0 (10)	1.09	3.11
RAL 315	16.0 (10)	1.92	9.56
RAL 301	22.1 (20)	0.95	5.17
w^{1118}	18.6 (20)	0.88	4.43
*Dm*2057	17.6 (20)	0.77	3.09
Nevada-04	18.4 (20)	0.93	4.92
Catalina Island (CA)	22.6 (20)	0.98	5.49
Santa Fe (NM)	22.5 (20)	0.90	4.64
Z32 (PK)	16.0 (20)	1.17	7.16
Z29	20.5 (20)	1.86	18.05
Z25	16.0 (20)	0.83	3.58
Z58	18.5 (20)	0.97	4.89
Z30	19.3 (20)	1.34	9.36
D. sechellia			
Robertson	7.6 (54)	0.27	1.04
w	7.6 (20)	0.50	1.31
NF31	8.2 (20)	0.53	1.61
NF49	8.2 (20)	0.38	0.77
An10	9.0 (20)	0.50	1.31
TMS1	8.9 (20)	0.50	1.29
LD12	8.0 (20)	0.39	0.79
NF72	7.5 (20)	0.44	1.00

Table 3.6 (continued): Mean adult ovariole number for *D. melanogaster* and *D. sechellia* populations. See Figure 3.7 for summary graphical representation of these data and statistical comparison of variance in adult ovariole number between species. The results of a Welch ANOVA (Welch *t*) analysis ($F_{1,18.53} = 33.1829$, $p < 0.0001$) indicate that the variance of the variances of ovariole number in *D. melanogaster* populations compared to *D. sechellia* populations are not homogeneous. We therefore used Bartlett's test, which does not make assumptions about the homogeneity of variances, to compare the variances between the two species (Figure 3.7).



Group	CV	Bartlett's Statistic	p
◆	11.1	7.1050	*0.013*
●	19.7	16.5806	*0.0007*
Dmel, ALL	15.9	14.8785	*0.0007*
Dsec, ALL	6.7		

Figure 3.7: Degree of plasticity correlates with relative interpopulational variation.

Range of ovariole number in different populations of *D. melanogaster* and *D. sechellia*. *CV*=coefficient of variation. Bartlett's test was used to compare variance of all *D. sechellia* populations to variance of indicated *D. melanogaster* groups. *p*-values indicating a significant difference are italicized. $n \geq 20$ for all genotypes.

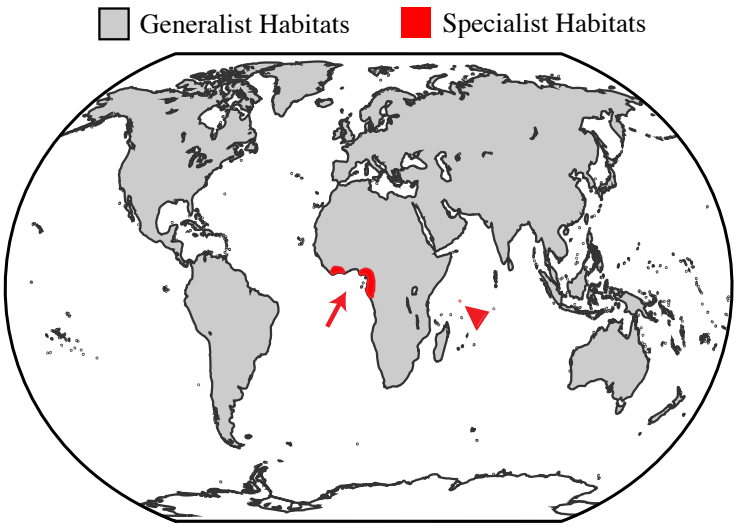


Figure 3.8: Global distributions of *Drosophila* species. Global species distributions of the generalists *D. melanogaster* and *D. simulans* (worldwide distributions: grey); the specialists *D. erecta* (red arrow: specializes on fruits of the genus *Pandanus* in west Africa) and *D. sechellia* (red arrowhead: specializes on the fruit *M. citrifolia* in the Seychelles, which is toxic to other *Drosophila* species) have limited habitats.

Correlation between ecology and nutritional plasticity of ovariole number

Finally, we asked whether nutrition-dependent plasticity of this critical reproductive trait was linked to broader ecological patterns of ovariole number diversity, which may indicate an adaptive value of nutritional plasticity. Mean ovariole number is correlated with nutritional host preference in many insect species from a range of global habitats (Fitt, 1990; Kambysellis and Heed, 1971; Leather et al., 1988). Specifically, species that have a wide host preference (generalists) or feed on abundant food sources, tend to have more ovarioles than species that feed on a restricted niche (specialists) or scarce food sources. This correlation has been used to support the idea of adaptive value of ovariole number in terms of *r*-*K* selection theory (Boulétreau-Merle et al., 1982; Kambysellis and Heed, 1971; Montague et al., 1981). Briefly, higher ovariole numbers permitting a larger number of offspring (*r*-selection) would be favored when host substrates are numerous and nutritionally rich, whereas restricted substrates would favor production of fewer offspring and hence decreased ovariole number (*K*-selection). Given our finding that mean ovariole number and nutritional plasticity of ovariole number are controlled by the same molecular mechanisms, we predicted that nutritional plasticity also correlates with range of host preference. Consistent with our hypothesis, we found that the cosmopolitan generalist species *D. melanogaster* and *D. simulans* (Figure 3.8) show high ovariole number plasticity and moderate body size plasticity in response to nutrition, while the specialist species *D. sechellia* (R'kha et al., 1991) and *D. erecta* (Lachaise et al., 1988) show low nutritional plasticity and no significant change in body size (Figure 3.9; Table 3.5). Because all species were reared on standard laboratory medium rather than native diets, we cannot rule out the possibility that our observed

ovariole numbers and associated phenotypes may be affected by the use of a standard, non-native diet that was necessary to allow us to make comparisons between species. We note, however, that in the case of *D. sechellia*, ovariole number reported here is the same as that reported when *D. sechellia* is reared on its host plant *Morinda citrifolia* (R'kha et al., 1991), suggesting that it may indeed be the degree of food source specialization, rather than a specific food source, which is the relevant parameter influencing ovariole number and its plasticity. Furthermore, low nutritional plasticity in *D. erecta*, which specializes on the non-toxic *Pandanus* genus of plants, indicates that this effect is not an artifact of the toxicity of *M. citrifolia* to other *Drosophila* species. Our experiments thus demonstrate that *Drosophila* species differ in their sensitivity to nutritional input, and suggest that relative IIS activity level may mediate these sensitivity differences.

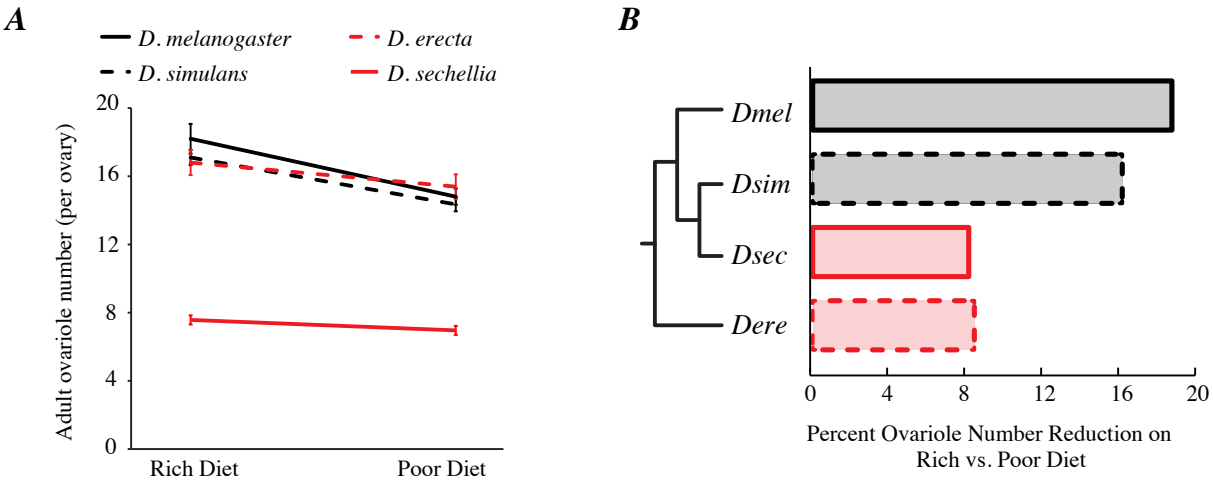


Figure 3.9: Degree of plasticity correlates with ecological niche. (*A*) Reaction norm of ovariole number on rich versus poor diet in four *Drosophila* species. Poor diet reduces ovariole number more in cosmopolitan generalist species (black lines) than in specialist species (red lines). Error bars show 95% CI of means. $n \geq 20$ for all genotypes and conditions. (*B*) The phylogenetic relationship between the four species analyzed is shown to the left of a bar graph showing percent ovariole number reduction on different diets.

Differences in plasticity lead to different relative ovariole numbers, and hence different relative reproductive capacities, between species in different environments (Figure 3.9A). These results imply that the degree of nutritional plasticity in ovariole number may be subject to selection, and has diverged across species in response to ecological niche. If specific nutritional plasticity is an adaptation to host preference range, then variation in IIS levels and sensitivity could provide a proximate mechanism for the observed correlation between mean ovariole number and host preference.

Discussion

Ovariole number is believed to be under stabilizing selection (Wayne and Mackay, 1998), and environmental changes cannot increase ovariole number beyond a lineage-specific maximum (Cohet and David, 1978; Engstrom, 1971). Evolution of reduced ovariole number has occurred convergently in many insect lineages (Green and Extavour, 2012), and is correlated with occupation of specialist ecological and nutritional niches (Fitt, 1990; Kambysellis and Heed, 1971; Leather et al., 1988). Consistent with these observations, we suggest that nutritional plasticity and reproductive capacity may present a tradeoff dependent on relative IIS activity: high IIS activity can increase mean ovariole number, but at the cost of strongly reducing ovariole number in poor nutritional conditions (Figure 3.9A). Because increased IIS also correlates with shortened lifespan (Clancy, 2001; Tatar et al., 2001), it is also possible that evolution of low plasticity due to low IIS levels could confer the advantage of an increased lifespan that is relatively robust to changes in nutritional conditions. Although we cannot yet determine which of these traits is the target of selection, we suggest that evolutionary diversification of both

ovariole number and its nutritional plasticity occurs through genetic changes that modulate IIS activity and sensitivity. Our data show that a functional consequence of evolutionary changes in IIS activity and sensitivity is modulation of plastic range between species, and that this range is correlated with interpopulational diversification. We previously showed that different developmental mechanisms, which are genetically separable, contribute to ovariole number evolution (Green and Extavour, 2012). We hypothesize that these alternate mechanisms may be targets of evolution for generating population-specific ovariole number while maintaining species-specific plastic responses.

While we have demonstrated that IIS has diverged between *Drosophila* species, what remains to be elucidated are the specific loci responsible for this divergence. Our data, particularly the interspecies hybrid complementation results, are consistent with the hypothesis that evolutionary change at the *InR* locus contributes to interspecies variation in ovariole number. Cross-species transgenesis and in-depth genetic analysis of IIS differences between species will be necessary to address this problem. We note here that both coding and noncoding differences exist between *D. melanogaster* and *D. sechellia* at the *InR* locus, none of which suggest obvious candidates for functional divergence. The protein coding sequences are 97% identical between these two species, and none of the amino acid changes occur within the known kinase domain. This suggests that slight structural or non-kinase-activity-related alterations in the *InR* protein could modulate signaling in such a way as to contribute to phenotypic change. Natural variation in a coding region indel polymorphism in *InR* among *D. melanogaster* populations is consistent with this hypothesis (Paaby et al., 2010).

IIS in multicellular animals is a conserved mechanism that coordinates cellular growth and proliferation with physiological condition, particularly nutritional state. The regulation of insulin/IGF signaling contributes to evolutionary change within invertebrate and vertebrate species (Emlen et al., 2012; Sutter et al., 2007). We have now shown that the regulation of IIS can underlie evolutionary morphological diversity both within and between species. Interestingly, evidence from functional studies in *D. melanogaster* and in horned beetles suggest that both increasing (Tang et al., 2011) and decreasing (Emlen et al., 2012) IIS can reduce nutritional plasticity. This suggests that IIS may be able to act as a nutritional stress response system that is either environment-sensitive or environment-insensitive. Ovariole number in *Drosophila* (this study) and ornament size in horned beetles (Emlen et al., 2012) appear to be examples of environment-sensitive nutritional stress responses, allowing generation of more offspring or exaggerated ornaments when food is plentiful, and restricting investment in these traits when food is scarce. An example of environment-insensitive nutrient stress response may to be external genitalia in *Drosophila*, which continue to devote resources to growth despite unfavorable environmental conditions (Tang et al., 2011). Given the wide conservation of IIS-mediated growth response, this work suggests a potentially pervasive role of plasticity in generating adaptive diversity.

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Chapter Four

Cross-species transgenesis to assess functional effects of divergence at the *Drosophila Insulin-like Receptor* (*InR*) locus between *D. melanogaster* and *D. sechellia*

Abstract

Insulin/insulin-like growth factor signaling (IIS) underlies both phenotypic plasticity and evolutionary diversification of ovariole number, a quantitative reproductive trait, in *Drosophila*. IIS activity levels and sensitivity have diverged between species, leading to both species-specific ovariole number and species-specific nutritional plasticity in ovariole number. Quantitative and developmental genetic analyses strongly support the hypothesis that evolution of the *Drosophila insulin-like receptor* (*InR*) gene, specifically, is at least partially responsible for this divergence. Phenotypic analyses and preliminary expression data suggest that evolution of the *InR* coding sequence is most relevant to differential function between species. This chapter details ongoing experiments to test this hypothesis explicitly via cross-species transgenesis, and describes anticipated results.

Introduction

Overview of IIS function

The insulin/insulin-like growth factor (IGF) signaling (IIS) pathway is an evolutionarily conserved pathway that controls growth, metabolism, aging and reproduction in animals (Britton et al., 2002; Broughton et al., 2005; Skorokhod et al., 1999) . Insulin/IGFs are circulated throughout the body to signal to body tissues nutritional condition (Britton et al., 2002; Broughton et al., 2005; Ikeya et al., 2002; Skorokhod et al., 1999) and physiological condition, including stress (Karpac and Jasper, 2009) and infection status (DiAngelo and Birnbaum, 2009; Dionne et al., 2006).[1] The IIS pathway is one of the primary mechanisms animals have evolved to coordinate systemic

[1] A more substantial discussion of the role of IIS in communicating nutritional condition appears in Chapter 3 of this thesis.

growth with environmental condition (reviewed in Mirth and Shingleton, 2012; Shingleton et al., 2007; 2008). Modulation of the pathway in specific organs leads to organ-specific growth control (Emlen et al., 2012; Tang et al., 2011).

IIS interacts with numerous signaling pathways to coordinate growth, proliferation, and differentiation. Cellular growth is coordinated through interaction with the target of rapamycin or TOR and AMPK pathways (reviewed in (Edgar, 2006). IIS interacts with the ecdysone signaling pathway to coordinate body and organ growth, and metamorphosis in insects (Colombani et al., 2005; Nijhout et al., 2006; Truman et al., 2006), and also to control germline stem cell niche differentiation (Gancz and Gilboa, 2013). Finally, in *Drosophila*, IIS interacts with EGFR (Ninov et al., 2009) and Notch (Hsu and Drummond-Barbosa, 2011; 2009b) pathways to coordinate growth and differentiation in a variety of cell types.

At the cellular level, IIS primarily controls cell-autonomous proliferation and growth. Several core molecular components of the pathway are remarkably well-conserved and appear to function similarly across animals (*Drosophila* gene names in italics): the peptide ligands (*Dilps*), the receptor tyrosine kinase *InR*, the receptor substrate *chico*, the lipid kinase *Pi3K92E* (*dp110*), the lipid phosphatase *pten*, the protein serine/threonine kinase *Akt*, and the transcriptional effector *foxo*. I will focus my subsequent discussions on the *Drosophila* pathway. IIS activation begins with the binding of *Drosophila insulin-like peptides* (Dilps) to INR. Eight Dilps have been described (Brogiolo et al., 2001; Slaidina et al., 2009) that all act through the single receptor InR. Dilp2 shares most amino acid sequence conservation (35%) with human (mature) insulin (Brogiolo et al., 2001). Chico phosphorylates Pi3k92E, among other targets, which in

turn phosphorylates the phosphatidylinositol second messengers. One action of the phosphoinositol-P3 messenger is activation of Akt, which goes on to phosphorylate a number of growth and proliferation targets. One effect of the IIS signal transduction cascade is the Akt-mediated phosphorylation of the transcription factor Foxo and its sequestration to the cytoplasm (Kramer et al., 2003; Puig, 2003). Unphosphorylated Foxo localizes to the nucleus and controls expression of many target genes that ultimately reduce cell proliferation (Puig, 2003). Thus IIS has the result of increasing proliferation. A second result is the phosphorylation of the ribosomal protein S6 kinase (S6K), which subsequently activates the protein translation machinery and hence promotes growth (Montagne et al., 1999).

Overview of InR *function and structure*

In *Drosophila*, *InR* is essential for normal development (Fernandez et al., 1995a). All described hypomorphic *InR* alleles are homozygous lethal; most animals die during embryogenesis or early larval stages (Brogiolo et al., 2001; Chen et al., 1996; Fernandez et al., 1995a). During embryogenesis, *InR* is required for central and peripheral nervous system development (Fernandez et al., 1995a; Pimentel et al., 1996), formation of the epidermis (Fernandez et al., 1995b), and specification of the correct number of somatic gonad precursor cells (Green and Extavour, 2012). Few transheterozygous combinations of weak alleles survive to adulthood. These animals phenocopy starvation phenotypes, showing strong developmental delay, reduced body size, increased lifespan, and sterility (Hsu and Drummond-Barbosa, 2009a; Kramer et al., 2003; Tatar et al., 2001; Zhang et al., 2009).

Four distinct transcripts are described for *InR* (Figure 4.1A), three of which have been previously characterized (Casas-Tinto et al., 2007). All four transcripts share exons 2-13. The initiator codon is in exon 3, and therefore all transcripts encode the same protein product and 3'UTR. Transcripts differ in the first exon of the 5'UTR. The *Drosophila* InR protein is similar in structure to the mammalian pro-insulin receptor and IGFR (Fernandez et al., 1995a). It is a large, 2,146-amino acid transmembrane tyrosine kinase receptor protein (Fernandez et al., 1995b) composed of 4 large subunits. The ligand-binding domain is found at the amino-terminus of the sequence, and the kinase domain at the carboxy-terminus. *Drosophila* INR contains a novel carboxy-terminal extension that may contribute to cell type-specific autophosphorylation (Fernandez et al., 1995a).

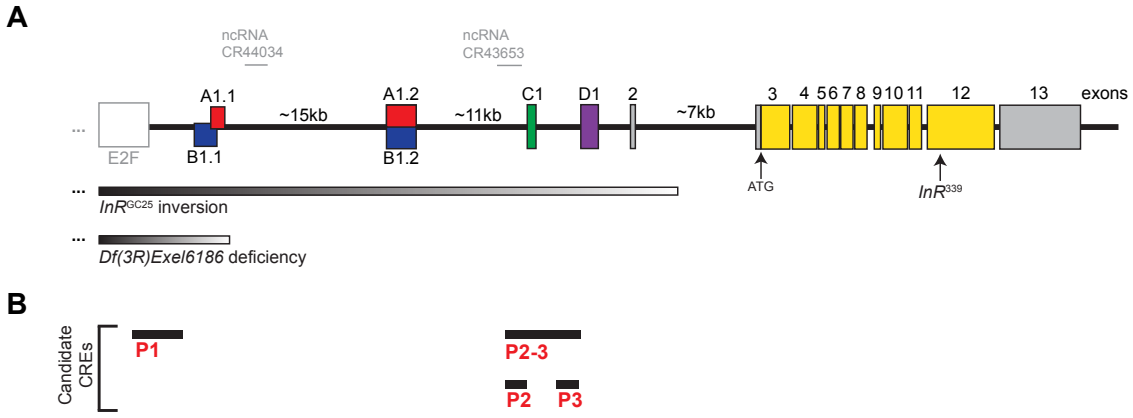

Figure 4.1: Schematic of the *InR* locus in *D. melanogaster*. (A) Exons of protein-coding genes are shown as solid-color boxes. Four distinct transcripts are derived from the *InR* locus. These transcripts differ by their first exon, each of which is shown as a different color (red, blue, green or purple). Two transcripts contain 13 exons (C and D). Two transcripts, A and B, encode two exons upstream exon 2 (A1.1 and A1.2; B1.1 and B1.2). Protein-coding exons are shown as yellow boxes. Non-protein-coding exons (UTRs) that are common to all four transcripts are shown as grey boxes. Putative non-coding RNA genes are indicated as grey lines. Locations of the loss of function mutations described in previous chapters are indicated either by an arrow (InR^{339}) or by gradient boxes (InR^{GC25} and *Df(3R)Exel6186*). The inversion breakpoints of InR^{GC25} are approximate (Chen et al., 1996). Ellipses at left of figure indicate continuation of chromosome/mutation. (B) Candidate cis-regulatory elements (here used synonymously with "promoters") are indicated as black boxes. The figure is approximately to scale.

InR *control of ovariole number determination*

Adult ovariole number is altered in *D. melanogaster* with loss of function mutations in *InR* and *chico* (Gancz and Gilboa, 2013; Green and Extavour, 2014; 2012; Tu and Tatar, 2003). Abrogation of IIS signaling throughout the body results in reduced ovariole number, demonstrating that IIS activity promotes ovariole formation. IIS is autonomously required within somatic ovarian cells to control their proliferation (Gancz and Gilboa, 2013; Green and Extavour, 2014). IIS, via *InR*, mediates nutritional control of ovariole number determination and plasticity (Green and Extavour, 2014).

Molecular Evolution of InR

IIS components are well conserved across the Drosophila phylogeny. The core components show evidence of purifying selection among 12 *Drosophila* species (Alvarez-Ponce et al., 2008). Component position in the pathway and strength of purifying selection are correlated, with upstream components (*InR* is considered the most upstream component) showing weaker purifying selection (Alvarez-Ponce et al., 2008). When looking for signatures of positive selection in *InR* among closely related Drosophilids, evidence of positive selection was identified within the cytosolic regions of the protein, which are predicted to contain the Dilp-binding domains (Guirao-Rico and Aguade, 2009). Analysis of nucleotide variation in the *InR* CDS from a derived European population of *D. melanogaster* did not reveal evidence of recent strong positive selection (Guirao-Rico and Aguade, 2009).

Natural Variation in InR

Disruptions in components of the IIS pathway lead to similar phenotypes (reviewed in (Edgar, 2006)). Natural variation in IIS components underlies variation in life history traits in different animals. Variation in Pi3k92E is associated with propensity to enter diapause in *Drosophila* (Williams et al., 2006). A coding indel polymorphism in *InR* is hypothesized to contribute to adaptation along a latitudinal cline in *D. melanogaster* (Paaby et al., 2010). These are important findings, as they strongly suggest that in natural contexts evolution at single loci can underlie complex transformations in several interrelated phenotypes. Moreover, several IIS components, including *InR*, *chico*, *PTEN*, *S6K*, *Dilps1-5*, *Pi3k92E*, and *Akt* are associated with chromosomal inversions that show latitudinal clines in frequency, and are hypothesized to contributed to natural variation in body size and development time (De Jong and Bochdanovits, 2003).

Experimental design and rationale

The most robust quantitative genetics analysis of interspecies ovariole number variation to date implicates *InR* as a locus of major effect (Orgogozo, 2006). However, despite fine scale mapping via selective phenotyping, the resolution of the QTL in this study is poor. A major effect size QTL is centered at cytological location 93D, almost the precise site of *InR*, which is found at 93E4-5. The 2-LOD supported interval of this QTL is 90D-93F1, which encompasses 400 predicted protein-coding genes. In Chapter 1 I showed that loss of function mutation of *InR* phenocopies essential differences in ovary development between *D. melanogaster* and *D. sechellia.* These results provided additional evidence to suspect *InR* as a causal locus of evolutionary genetic change. In

Chapter 3 I provided a more specific test of this hypothesis using interspecies hybrid genetic complementation. I showed that in a common genetic background, INR from each species causes species-specific phenotypes. In this chapter I describe the efforts I have made thus far to perform cross-species transgenesis in order to test explicitly the hypothesis that ovariole number divergence between *D. melanogaster* and *D. sechellia* is due, in part, to evolution of *InR*.

Neither the *InR* protein-coding sequence nor the putative promoter sequences are identical between these two species, leaving open the question of whether cis-regulatory or coding mutation controls interspecies divergence. Hybrid complementation analyses also leave this question open, as both coding (InR^{339} is a non-synonymous coding point mutation) and non-coding (InR^{GC25} is an inversion mutation within the putative *InR* upstream regulatory sequence that removes portions of the 5'UTR; *Df(3R)6186* is a deficiency that deletes portions of the 5'UTR) mutations show terminal filament (TF) number reduction phenotypes in hybrids between *D. melanogaster* and *D. sechellia* (Green and Extavour, 2014). Despite this, indirect evidence suggests that the difference relevant to interspecies ovariole number divergence is a protein-coding change.

First, in a heterozygote containing the InR^{GC25} inversion allele, INR protein expression was shown to be reduced to almost 50% of wild type levels, however, insulin kinase activity was not significantly changed from wild type levels (Chen et al., 1996). Adult ovariole number in InR^{GC25} heterozygote females is the same as in wild type females (Green and Extavour, 2014). These results are consistent with the hypothesis that relative INR activity and somatic ovarian cell proliferation are not straightforward consequences of relative INR protein expression level. Moreover, Nuzhdin et al.

(Nuzhdin et al., 2009) measured covariance between transcript expression of IIS components and mean trait value of IIS-mediated phenotypes (lifespan, oxidative stress, body size, starvation, development time, and desiccation resistance) in different genotypes. They found that *InR* transcript expression level was not significantly correlated to any of the examined traits.

I additionally assayed *InR* transcript expression from both individual species via qPCR. I did not obtain consistent results across seven experimental trails. These results are summarized in Table 4.2. I most often observed *InR* expression in whole female larvae of *D. sechellia* to be no different or slightly higher than of *D. melanogaster* (Table 4.2). I believe the inconsistency in results is attributable to relatively low expression levels of the *InR* transcript. It is also possible that the assays I have performed so far are not at high enough resolution to capture potentially rapid dynamics of *InR* transcription (Puig and Tjian, 2005). However, I previously demonstrated that IIS activity reporters consistently show that IIS activity is higher in whole females and larval ovaries of *D. melanogaster* compared to *D. sechellia* (Green and Extavour, 2014). This further suggests that if *InR* mediates IIS activity difference, it may not be at the level of mRNA transcription.

Table 4.1: Summary of qPCR trials/experiments to assay *InR* expression in wild type *D. melanogaster* (Oregon R) and *D. sechellia*.

Trial	n^a	Ore R Fold Change	Lower Error Bound[b]	Upper Error Bound	Ds Fold Change[c]	Lower Error Bound	Upper Error Bound	Primers, Notes[d]
1	3	1	0.39	2.55	0.87	0.19	3.92	'InR-1'; coding region
2	3	1	0.40	2.53	0.66	0.07	6.51	'InR-1'
3	3	1	0.62	1.60	1.22	0.80	1.86	'InR-1'
4	3	1	0.77	1.30	1.75	1.29	2.37	'InR-1'
5	3	1	0.26	3.81	1.15	0.17	7.81	'InR-2'; exon-exon junction overlap
6	3	1	0.59	1.70	1.16	0.73	1.86	'InR-2'
7	**10**	**1**	**0.64**	**1.69**	**5.22**	**5.20**	**5.26**	**'InR-2'**

Table 4.1: Summary of qPCR trials/experiments to assay *InR* expression in wild type *D. melanogaster* (Oregon R) and *D. sechellia*. In only one experiment was expression level shown to be significantly different between *D. melanogaster* and *D. sechellia*, which is indicated in **boldface** text. [a] *n* refers to the number of biological replicates, per species, in a particular experiment. A biological replicate consisted of 10 well-fed wandering third instar female larvae. [b] Error bounds are one standard deviation. [c] Fold change was calculated using the $\Delta\Delta C_T$ method. Fold change values are normalized to Oregon R values for a particular trial/experiment. [d] These primer pairs are listed in Table 4.2.

Table 4.2: List of primers used for indicated cloning and qPCR experiments.

ID	Primer Sequence (5' → 3')	Notes
HA-tagged *InR* CDS inserted into pVALIUM22 (CPEC cloning)		
1xHA tag + InR CDS forward	ATGTACCCATACGATGTTCCTGACTATGC GTTCAATATGCCACGGGGAGTG	• Successfully cloned *InR* from both species with 3xHA tag, but fragments never successfully cloned into pVAL22
InR CDS reverse	CTCCGATGTCTCGCCTGAATTCTTACGCC TCCCTTCCGATGA	
pVal22 forward	GAATTCAGGCGAGACATCGGAG	
pVal22 reverse	CATAGTCAGGAACATCGTATGGGTACAT CATGCTAGCGGCTGAATATGGGATG CATAGT	
3xHA tag + InR CDS forward	GAATATGCTAGCATGTACCCATACGATGT TCCTGACTATGCGGGCTATCCCTATGACG TCCCGGACTATGCAGGATCCTATCCATAT GACGTTCCAGATTACGCTTTCAATATGCC ACGGGGAGTG	
***Dmel* and *Dsec* regulatory constructs inserted into 1xHA-*InR*.pVALIUM22 (CPEC)**		
P1 forward	GTATGCTATACGAAGTTATCTGCAGGCA GGTCCGAATGTCTACTGTTTCAATTAAAC TG	
P1 reverse	GTCGACGAGTCTCCGCTCGGA CCAGCCGTTACCCTTGTTGTAT	
P2 forward (FOXO-responsive element A)	GTATGCTATACGAAGTTATCTGCAGGCA GGTCGCCTTTGTTATCGATAGGTTCG	
P2 reverse (FOXO-responsive element A)	GTCGACGAGTCTCCGCTCGGA CGAGGCACAAAAGAACCTAATC	
P3 forward (FOXO-responsive element B)	GTATGCTATACGAAGTTATCTGCAGGCA GGTCCTTTTGTTTCGCCATGCACTTTTC	
P3 reverse (FOXO-responsive element B)	GTCGACGAGTCTCCGCTCGGA GTGCAATGACATTTTCAAGTGCC	
pVal22 forward	GACCTGCCTGCAGATAACTTCGTATAGCA TAC	
pVal22 reverse	TGTCCTCCGAGCGGAGACTCGTCGAC	
Hsp70 promoter inserted into 1xHA-*InR*.pVALIUM22, replacing P-Transposase (CPEC)		
Hsp70 promoter forward	GAGCGCCGGAGTATAAATAGAGGCG	
Hsp70 promoter reverse	TATTCAGAGTTCTCTTCTTGTATTCAATA ATTACTTCTTGGCAG	
pVal22 forward	CGCCTCTATTTATACTCCGGCGCTC GTCGACGAGTCTCCGCTCGGA	

Table 4.2 (Continued): List of primers used for indicated cloning and qPCR experiments.

pVal22 reverse	CTGCCAAGAAGTAATTATTGAATACAAG AAGAGAACTCTGAATA TCCCATATTCAGCCGC	
1xHA-tagged *InR* inserted piecewise into pVALIUM22		
Ds *InR* part 1 forward	CATGCAATTTCGAGCTGCAAGA	
Ds *InR* part 1 reverse	TCTTGCAGCTCGAAATTGCATG	
Ds *InR* part 2 forward	CCGATGGACGCGGATAAATATG	
Ds *InR* part 2 reverse	CTCCGATGTCTCGCCTGAATTC CATATTTATCCGCGTCCATCGG	
Ds *InR* part 3 forward	CTCCGATGTCTCGCCTGAATTC CATGCAATTTCGAGCTGCAAGA	
Ds *InR* part 3 reverse	CATATTTATCCGCGTCCATCGG	
***InR* qPCR primers**		
'InR-1' forward	ACGCTTTGGACGGCGACAGG	
'InR-1' reverse	CTAATGGCCATACCGCTGCC	
'InR-2' forward	GTGAAAGCGGCGCTCACGT	
'InR-2' reverse	CATATTTATCCGCGTCCATCGG	

I hypothesize that protein-coding changes in *InR* are the principal determinants of IIS activity difference between *D. melanogaster* and *D. sechellia.* As such, the major goal of the cross-species transgenesis experiment is to test *in vivo* functional differences of the InR protein from *D. melanogaster* and *D. sechellia*. Nevertheless, I have designed experiments to directly test non-coding contribution by using regulatory information from each species to drive the coding sequence from each species.

The *InR* locus in *Drosophila* is complex, including multiple transcriptional start sites, putative transcription factor binding sites, and two predicted non-coding RNA genes. The entire locus, including 5'UTR, CDS, 3'UTR and intervening intergenic sequence, spans ~50kb (Figure 4.1A). Relatively little is known of what sequence may be relevant for controlling *InR* expression. Few specific sequences within the *InR* promoter have been demonstrated to be sufficient to direct FOXO-dependent *InR* expression (Casas-Tinto et al., 2007; Puig, 2003). Because of the complexity of the locus and the knowledge of these specific regulatory sequences, I chose to begin my analysis with a more targeted approach rather than simply working with the several kilobases of sequence upstream of the most upstream transcription start site. I have approached generating constructs in two phases:

- In 'Phase 1,' I am generating UAS expression constructs for the *InR* CDS from both *D. melanogaster* and *D. sechellia*. This involves introducing the *InR* CDS into the pVALIUM22 expression vector for site-specific integration into the *D. melanogaster* genome (specifically onto chromosome II for my purposes). Flies containing these constructs can be crossed to an *InR*-GAL4 line to generate

species-specific *InR* (over)expression in *D. melanogaster* in an *InR*-null background.

- In 'Phase 2,' I am replacing the UAS sequences with the specific regulatory sequences of the *InR* promoter identified to be sufficient to direct FOXO-dependent *InR* expression. I will do this in a combinatorial manner, such that promoters from each species will drive the *InR* CDS from each species. Flies containing these constructs can be introduced into a *D. melanogaster InR*-null background to generate species-specific *InR* expression.

Materials and Methods (including brief discussion)

RNA extraction/cDNA synthesis

Total RNA was extracted from well-fed female larvae and adult flies that were grown on rich diet. RNA was extracted using Trizol (Invitrogen), treated with TURBO DNase-I (Ambion, Life Technologies), and phenol-chloroform extracted. cDNA was prepared using oligo-dT primers and 0.5-1µg RNA per reaction with Superscript III First Strand Synthesis Kit (Invitrogen).

Cloning InR *coding sequences.*

After several failed attempts at NheI/EcoRI restriction digest and isothermal ligation cloning (Gibson et al., 2010; 2009), circular polymerase extension cloning (CPEC) (Quan and Tian, 2009) proved most useful for this cloning given the relatively large size of the components. The primers used for cloning each component are listed in Table 4.2.

Successfully cloning the full-length *InR* coding sequence presented several challenges. Special conditions were required to obtain the full-length transcript at even low levels. Full-length *InR* transcript was only obtained using oligo-dT-primed cDNA, and not when using random hexamer primers. I suspect that this is due to two reasons. First, the *InR* transcript may be in relatively low abundance. Selectively amplifying poly-A mRNAs thus increases its relative abundance within the cDNA pool. Second, priming by random hexamers biases for shorter cDNA products. However, the *InR* transcript is ~7kb in both species, which is quite large for standard PCR amplification. Furthermore, the full-length *InR* transcript could only be cloned under GC-rich conditions when using *Phusion Hot-start* Taq polymerase, despite the fact that no obvious GC-rich regions appear within the *InR* transcript of reference *D. melonagster* and *D. sechellia* genomes. Standard conditions were sufficient when using Advantage 2 polymerase.

Cloning of candidate promoters

Genomic DNA was extracted from *D. melanogaster* Oregon R and *D. sechellia* Robertson adult females using the Qiagen genomic DNA extraction kit (Ambion, Life Technologies). Identical primers were designed to be able to be used with both species. Orthologous *InR* promoter sequences were identified using the multigenomic DNA sequence analysis tool EVOPRINTER-HD (Odenwald et al., 2005). EVOPRINTER-HD uses pair-wise BLAT to identify conserved nucleotides shared between a reference sequence and a set of orthologous genome sequences.

Generating expression constructs

The pVALIUM22 expression vector was used as the base vector for this work (Figure 4.2). pVALIUM22 was originally designed to induce robust expression of RNAi hairpins in the germline and soma (Ni et al., 2008; 2007). Transgenes are inserted at known genomic loci via ΦC31 site-specific integration. The specific elements of the vector are described in Figure 4.2. I also generated a new vector based on pVALIUM22 in which the P-Transposase promoter is replaced with the Hsp70 promoter from *D. melanogaster* Oregon R. The Hsp70 promoter drives higher expression in the soma compared to the P-Transposase promoter (Ni et al., 2011).

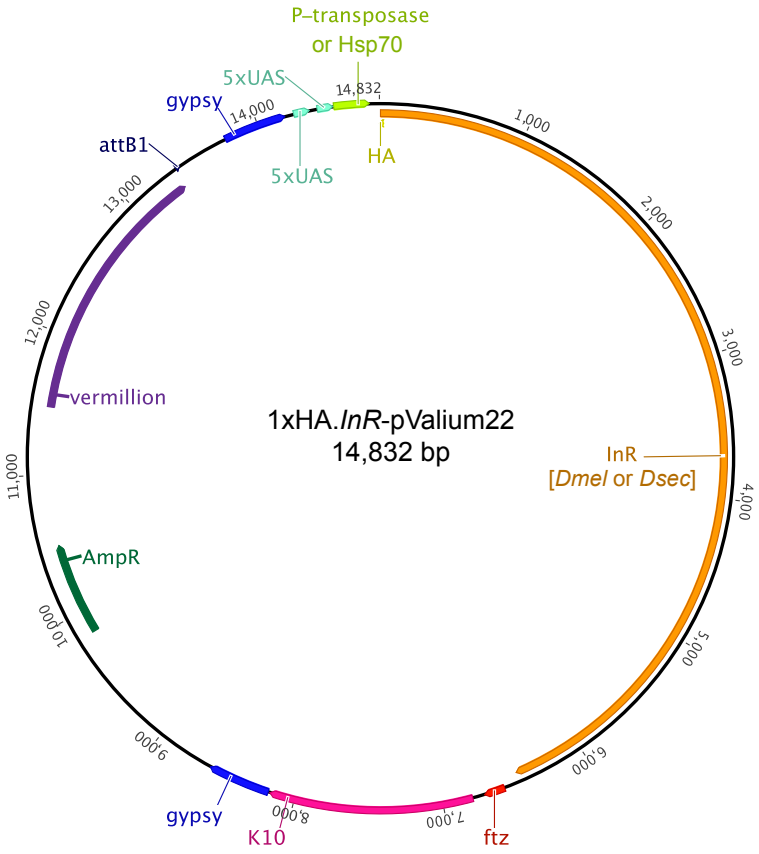

Figure 4.2: Structure and components of UAS-*InR* expression constructs.

pVALIUM22 components: '5xUAS' (light blue): tandem repeats of the GAL4-responsive UAS sequence; 'P-transposase' (light green): ubiquitous promoter; 'HA' (yellow): ten amino acid HA sequence; 'InR' (orange): *InR* CDS; 'ftz' (red): intron to permit efficient transcription; 'K10' (pink): contains poly-A transcription termination signal and transport/localization elements that promote efficient expression in the germline; 'gypsy' (blue): an insulator; 'AmpR' (dark green): bacterial selection antibiotic (ampicillin); 'vermillion' (purple): dominant visible marker (vermillion eye color) for selection of transformants in flies. 'attB1' (navy blue): target sequence for site-specific integration into *Drosophila* genome. I am also generating constructs in which the P-transposase promoter is replaced by the Hsp70 promoter (from Oregon R).

Results

Comparative Analysis of Promoter Sequences

Putative *InR* promoter sequences are highly conserved between *D. melanogaster* and *D. sechellia*, although not identical (Figure 4.3). The three promoters are not conserved to the same extent. P2 overlaps a predicted non-coding RNA gene (CR43653) (Figure 4.1A), which has yet to be validated or characterized. The higher sequence conservation observed in P2 compared to P1 and P3 may thus reflect increased conservation of this potentially functional RNA. P1 shows least sequence conservation of the three promoters.

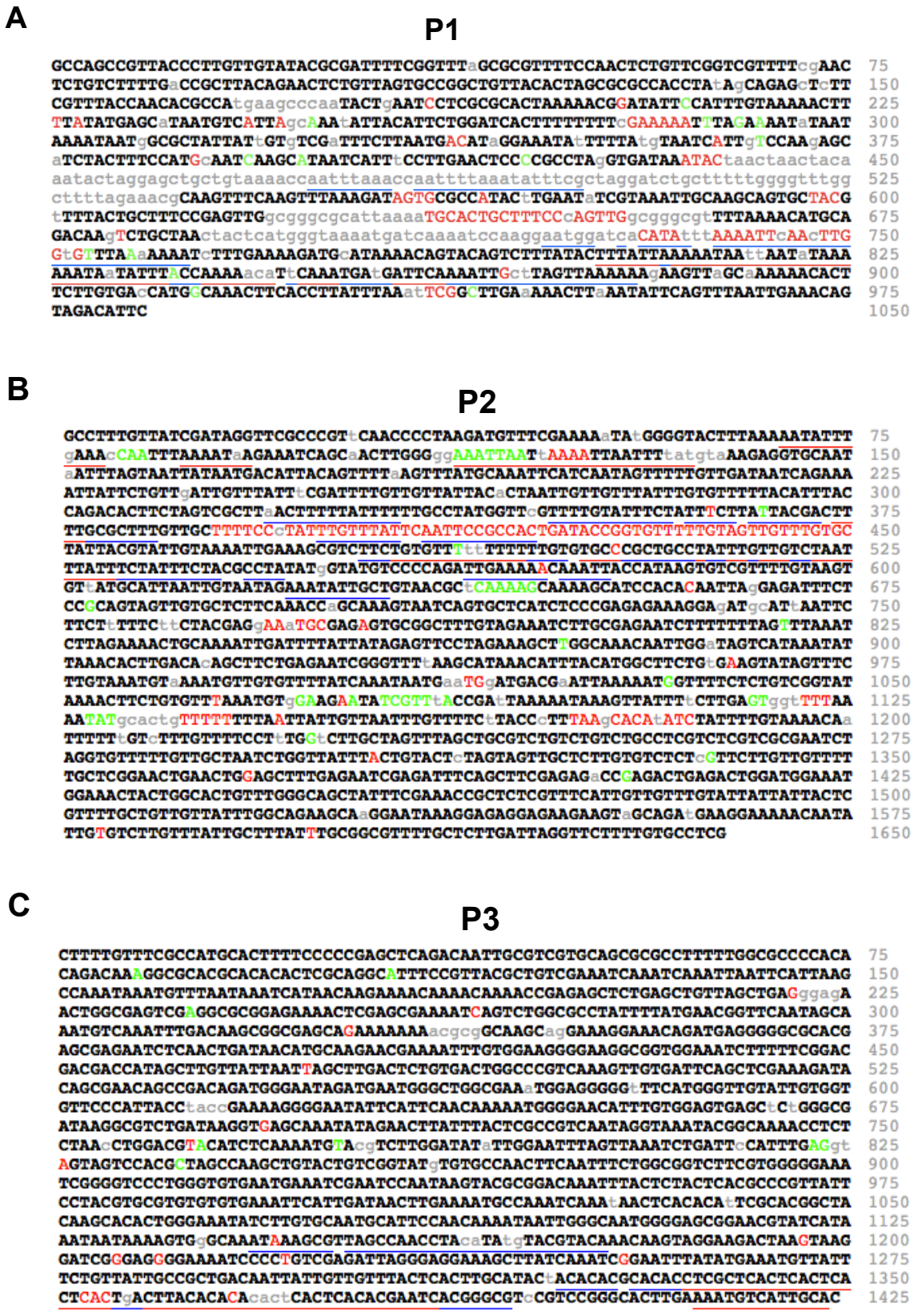

Figure 4.3: EVOPRINTER-HD analyses of candidate promoter regions.

Figure 4.3 (continued): EVOPRINTER-HD analyses of candidate promoter regions. EVOPRINTER-HD analysis was preferred over traditional BLAST because of the ease with which it identifies and visually displays conserved sequence. In this figure orthologous sequences from *D. melanogaster*, *D. sechellia*, and *D. simulans* are compared. Putative promoter sequences are identified as labeled in Figure 4.1B: (A) P1, (B) P2 and (C) P3. **BOLDFACE CAPITAL** letters indicate conserved sequence between all three species. Grey lowercase letters show sequence that differed between all three species. Underlined sequence (red or blue) indicates potentially repetitive sequence. In (A), letters in **RED** indicate sequence that is identical between *D. melanogaster* and *D. simulans*, but differs in *D. sechellia*; letters in **GREEN** indicate sequence that is identical between *D. melanogaster* and *D. sechellia*, but differs in *D. simulans*. In (B) and (C), the colors are reversed. (The program does not allow control of colors.)

Cloning Progress

Full-length *InR* sequences from *D. melanogaster* and *D. sechellia* were successfully amplified. For each sequence, an N-terminal single copy hemagglutinin (HA) tag was included for subsequent immunodetection with an anti-HA antibody. Attempts were made with 3x-HA tags, however, these constructs were not successfully cloned into pVALIUM22. *D. melanogaster InR* was cloned into pVALIUM22 (referred to as UAS-InR^{Dmel}) and the Hsp70 promoter-modified pVALIUM22 (referred to as UAS^{Hsp70}-InR^{Dmel}). All attempts to clone the full-length *D. sechellia InR* into pVALIUM22 failed. Because CPEC assembly allows for the assembly of sequences without "scarring," piecewise assembly of the *D. sechellia InR* is underway, due to my hypothesis that the length of the fragment is a significant obstacle.

Five of 8 promoter constructs have been successfully amplified under my new cloning scheme, which includes modified primers for CPEC assembly (Table 4.2). Previous amplification of these sequences suggests that the sequences are of similar sizes from both species, as is expected from the EVOPRINTER and BLAST analyses.

Genetic Analysis Design and Interpretation of Anticipated Results

UAS overexpression constructs

The UAS overexpression constructs (collectively referred to as UAS-*InR*) will test for functional differences in the INR protein from *D. melanogaster* and *D. sechellia*, albeit not necessarily at physiologically relevant expression levels. *InR*-GAL4 lines generated by P-element insertion into the *InR* locus exist in the Bloomington Stock Center. These drivers may be expected to drive physiologically relevant *InR* expression

levels, and thus would be ideal, although these lines require validation. This would require crossing UAS-*InR* and *InR*-GAL4 driver chromosomes (all on chromosome II) into *InR*-deficiency backgrounds (*InR* null/hypomorphic mutant balanced over a chromosome with a ubiquitously expressed fluorescent marker, in order to distinguish larval-pupal transition (LP) stage larvae if experimental crosses are lethal prior to eclosion). I would do this experiment in homozygous *InR* null mutant backgrounds.

If flies from these crosses survive to adulthood, and individual ovarioles are readily separable, ovariole number can be counted. Otherwise, LP-stage TF number can be counted. If my hypothesis that the INR protein from *D. melanogaster* confers greater IIS signaling than from *D. sechellia* is correct, I would expect that expression of UAS-InR^{Dmel} will yield females with greater ovariole number, body size, and nutritional plasticity of ovariole number compared to expression of UAS-InR^{Dsec}.

Furthermore, it will be useful to test these constructs for ovary-specific growth and proliferation effects with the *c587-GAL4* driver, which drives expression specifically in somatic cells of the larval ovary beginning in third instar (Manseau et al., 1997). I and others (Gancz and Gilboa, 2013) have previously demonstrated *InR* loss- and gain-of-function phenotypes using this driver. This experiment would also be done using *InR*-deficiency backgrounds as previously described. Similar to above, I would expect that expression of UAS-InR^{Dmel} will yield females with greater ovariole number compared to expression of UAS-InR^{Dsec}.

Species-specific, FOXO-responsive promoter constructs

The species-specific promoter constructs (collectively referred to as P_n^x-InR^x) will test for functional differences in the INR protein from *D. melanogaster* and *D. sechellia* at what may be closer to physiologically relevant expression levels (Figure 4.4). It is important to note, though, that the assays conducted in Puig et al. (2003) and Casas-Tinto et al. (2007) do not give any indication as to what extent wild type expression levels are recapitulated by the specific sequences identified in their studies. These authors tested each promoter sequence individually. I have also amplified these sequences individually, but also amplified a contiguous sequence containing both promoter domains ('P2-3^x'; Figure 4.1A, Figure 4.4). Finally, I also amplified a previously untested sequence upstream a distant transcript start site ('$P1^x$'; Figure 4.1A, Figure 4.3A, Figure 4.4). This sequence is uncovered in the *Df(3R)Exel6186* deficiency mutation and also contains a *Bab1* binding site. *Bab1* is specifically expressed in TF cells in the larval ovary (Couderc et al., 2002). Comparison of the $P3^{Dmel}$-InR^{Dmel} homozygote (in a homozygous *InR*-null background) to wild type (*InR*-null heterozygote, which has wild type ovariole number) would determine the extent to which the two FOXO-responsive promoter elements recapitulate wild type expression.

Similar to the UAS constructs, if flies from these crosses survive to adulthood, and individual ovarioles are readily separable, ovariole number can be counted. Otherwise, LP-stage TF number can be counted. If my hypothesis that the INR protein from *D. melanogaster* confers greater IIS signaling than from *D. sechellia* is correct, I would expect that when comparing expression by the same promoter construct, expression of P_n^x-InR^{Dmel} will yield females with greater ovariole number, body size, and

nutritional plasticity of ovariole number compared to expression of P_n^x-InR^{Dsec}. This should be the case regardless of the species-specific promoter construct used.

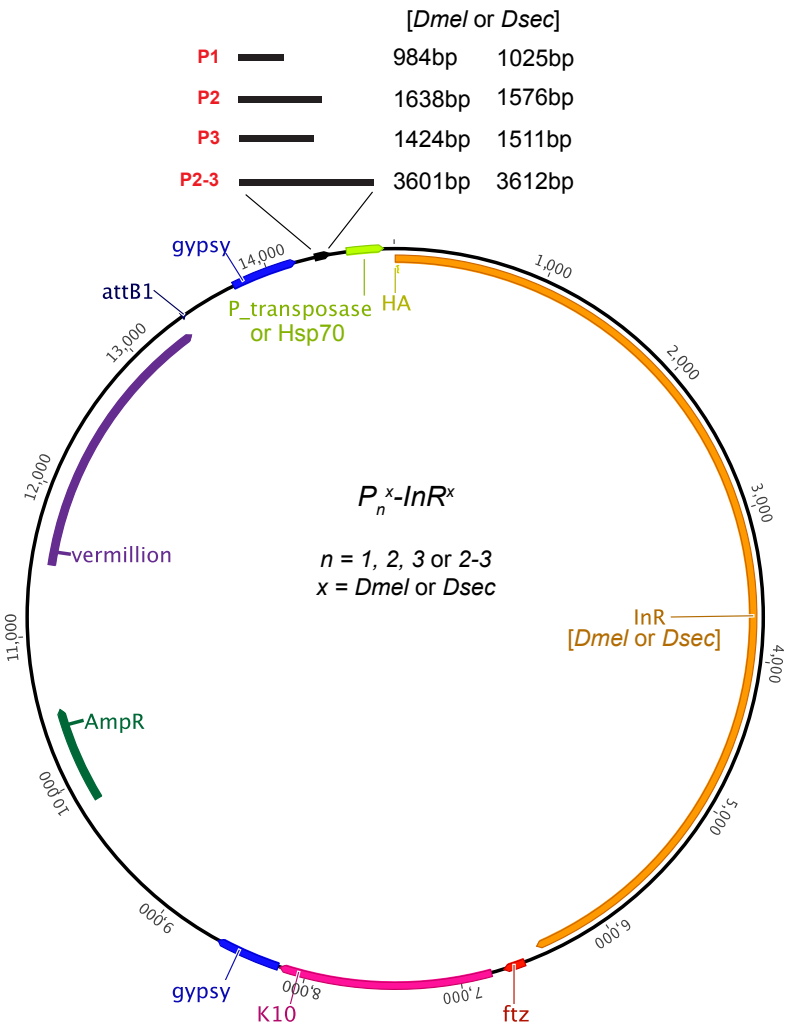

Figure 4.4: Structure and components of species-specific promoter-regulated expression constructs. Vector components are as in Figure 4.2. The sizes of putative promoter sequences for both species are indicated.

Discussion

Although these experiments are at their beginning stages, I do want to consider briefly the implications of evolution at the *InR* locus. A critical determinant of the repeatability or predictability of genetic evolution is the degree and consequence of pleiotropy. Pleiotropy is classically interpreted in a gene-centered fashion, however, it is more usefully considered in a mutation-centered view, as different mutations in the same gene can have different pleiotropic effects (Hoekstra and Coyne, 2007; Linnen et al., 2013; Martin and Orgogozo, 2013). Mutations with strong pleiotropic effects are expected to be disfavored in adaptive evolution (reviewed in (Orr, 2005)). What is the fate, then, of genes such as *InR* that have highly pleiotropic effects? Modularity via cis-regulatory evolution is a popular response, particularly with respect to morphological evolution (Carroll, 2008; Martin and Orgogozo, 2013; Stern and Orgogozo, 2009). In this way, the essential function and structure of these genes can be preserved while spatiotemporal expression may be modulated to achieve new form/function. As aforementioned, however, I predict that the changes relevant to ovariole number divergence between *D. melanogaster* and *D. sechellia* are primarily the result of coding sequence differences between these two species. This work may provide an interesting model for understanding the evolution of highly pleiotropic genes/mutations.

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Chapter Five

Discussion

Discussion

Each preceding chapter includes a discussion of the specific data presented therein. I use this final chapter to discuss this work in total, specifically with respect to the contributions of the *Drosophila* ovariole number model to the Quantitative Trait Gene (QTG) program. I also present future directions that this work may take, some of which are in progress in the lab.

Contribution to the Quantitative Trait Gene Program

In my opinion, the *Drosophila* ovariole number model makes two significant contributions to the QTG program. First, this model gives insight into the evolutionary dynamics of "complex" traits. My determination of ovariole number as a "complex" trait, an admittedly poorly defined category, is based on the following: 1) both ovariole number determination and variation are polygenically controlled; 2) distinct developmental mechanisms, which involve different cell types and occur across different life stages, control ovariole number determination and variation; and 3) ovariole number is phenotypically plastic. Second, this model is an attractive candidate for a highly integrative "eco-evo-devo" model.

Evolutionary dynamics of complex traits

This work highlights considerations that come to bear on the rate and course of evolutionary innovation. First is the contribution of phenotypic plasticity to heritable diversification. This topic is treated thoroughly in Chapter 3, and thus will be discussed

briefly in a different context here. Second are the implications of polygenic trait determination to trait evolution.

Contribution of Phenotypic Plasticity to Heritable Evolution

Orgogozo and Stern observed different patterns of genetic evolution between short-term (intraspecies) and long-term (interspecies) evolution (Stern and Orgogozo, 2009). They suggest that evolution over shorter timescales may result from mutations with stronger pleiotropic, epistatic, and/or plastic effects, whereas evolution between species may result from more specific mutations that have relatively little or no epistatic, pleiotropic or plastic effects. My data suggest that there is a difference in how intraspecies versus interspecies variation in ovariole number occurs (Figure 2.18). I reiterate that I have not identified the specific genes that control difference between these different lineages. However, it is likely that different genetic mechanisms control the different developmental mechanisms that lead to convergent phenotypes, and I have described strong candidates for each (Chapter 2). At least among the lineages I analyzed, intraspecies variation in ovariole number occurred through differences in larval ovary morphogenesis late in larval life, while interspecies variation occurred through differences in SGP specification in embryogenesis and subsequent proliferation of somatic cells throughout larval life (Figures 2.18, 2.19).

Interestingly, my data are not fully consistent with the expectation of Orgogozo and Stern. My work complicates their hypothesis of how evolution is expected to proceed on different time scales. Evolution via a modular cis-regulatory element (CRE) is taken as the classic example of a fine-tuned, long-term (interspecies) evolutionary strategy (Carroll, 2008; Stern and Orgogozo, 2009). *bab* is a homeotic regulator of several traits

related to reproduction, including ovariole formation (Godt and Laski, 1995; Green and Extavour, 2012; Sahut-Barnola et al., 1996), sex comb bristle number determination (Couderc et al., 2002; Godt et al., 1993), and abdominal bristle and trichome patterning (Couderc et al., 2002; Kopp et al., 2000). In one case, *bab* has been demonstrated to underlie evolutionary diversification of a trait. Sexually dimorphic *bab* expression is controlled by a distinct CRE within the *bab* promoter region that has diverged among different *Drosophila* lineages, leading to different patterns of sexually dimorphic abdominal pigmentation across species (Kopp et al., 2000; Williams et al., 2008). However, I found in my work (described in Chapter 2) that loss of function of the transcription factor *bab2* affected larval ovary morphogenesis without disrupting SGP establishment, mimicking the developmental basis for ovariole number within the species *D. melanogaster*.

In contrast, loss of function of *InR* results in reduced somatic gonad precursor (SGP) establishment and somatic cell proliferation late in larval life, phenocopying the developmental differences seen between the species *D. melanogaster* and *D. sechellia* that resulted in differing ovariole number between species. As demonstrated in this thesis, *InR* mediates nutritional plasticity in *Drosophila*. Furthermore, *InR* mutations are highly pleiotropic, as disruption of *InR* is known to cause a suite of correlated phenotypes, including longevity, reduced fecundity, and smaller body size (Tatar et al., 2001). Natural allelic variation in *InR* concomitantly affects oxidative stress, chill coma recovery and fecundity in ways consistent with predicted patterns of selection along longitudinal clines (Paaby et al., 2010). As argued in Chapter 3, it may be the case that global *plastic* response controlled by IIS actually promotes evolutionary diversification by providing a

range of phenotypes whose underlying genetic variation can be subject to selection by genetic accommodation, genetic assimilation or other means. Moreover, the *pleiotropic* response governed by IIS may promote evolutionary diversification by providing multiple phenotypes, for example plasticity, fecundity, stress tolerance, longevity, etc., on which natural selection can act.

Polygenic Trait Determination May Promote Rapid Trait Evolution

Quantitative genetic analyses for variation in ovariole number (reviewed in Chapter 1) did not always identify fully concordant loci. My work implies that the different results found in different QTL analyses reflect actual biological differences (e.g. interspecies versus intraspecies mapping or gene-by-environment interaction under different nutritional regimes). I show in Chapter 2 that convergent ovariole numbers in different *Drosophila* lineages are generated through distinct developmental mechanisms, and that these mechanisms are genetically separable. This strongly suggests that a broad spectrum of the genetic loci that control ovariole number determination also underlie natural variation of the trait. This would be considered by some a surprising result, as it does not follow the hypothesis that phenotypic evolution tends to occur at genetic hotspots at various genetic resolutions and across various phylogenetic distances (Conte et al., 2012; Martin and Orgogozo, 2013; Stern and Orgogozo, 2009). Additional examples of convergent evolution of similar traits through different genetic mechanisms have been shown to occur (Protas et al., 2011; Roelants et al., 2010; Shapiro et al., 2009). Why should this be the case? Are some traits, such as ovariole number, simply

exceptional? Or is there some biological reason why evolution should take the same route in some cases but not others?

One hypothesis is that traits that can be changed in multiple ways, through different developmental and/or genetic mechanisms, have a larger effective search space from which to identify adaptive solutions. Trait determination is, in effect, modularized at the level of developmental mechanism versus at the level of cellular differentiation (for example, via spatiotemporal control of transcription factor expression). This has the potential effect of speeding trait evolution. It is interesting to consider that reproductive traits often evolve rapidly, hence showing substantial diversity among closely related lineages (Couderc et al., 2002); ovariole number follows this trend (Figure 2.2). Convergent evolution of similar phenotypes through distinct developmental mechanisms has been reported for a number of traits, including *Drosophila* sex combs, a reproductive trait (reviewed in (Kopp, 2011)), and sexually dimorphic skull shape across species of *Anolis* lizards (Sanger et al., 2013), organisms famous for their rapid adaptive radiation across the islands of the Greater Antilles (Losos, 2009). QTL analyses of *Drosophila* sex combs reveal that multiple small-effect loci contribute to intraspecies variation in sex comb size (Kopp, 2011). Few large-effect and several small-effect loci contribute to interspecies sex comb variation. Resolution of these studies is not sufficiently high to determine the extent of overlap between these sets of loci. The genetic basis of *Anolis* skull shape variation has yet to be reported.

Ovariole number in Drosophila *as an integrative "eco-evo-devo" model*

Several models have emerged with the objective of identifying the genetic basis of ecologically relevant adaptations (reviewed in (Hoekstra and Coyne, 2007; Nadeau and Jiggins, 2010; Stapley et al., 2010). In a few remarkable cases, the specific nucleotide changes that have evolved and are causally linked to adaptive phenotypic change have been identified and functionally demonstrated, including coat color in beach mice (Hoekstra, 2006), pelvic evolution in stickleback fish (Chan et al., 2010; Shapiro et al., 2006; 2004), pigmentation in *Drosophila* (Rebeiz et al., 2009; Wittkopp et al., 2009), and host sterol specialization in *Drosophila pachea* (Lang et al., 2012). Studies such as these are critical for the development of a more complete theory of genetic evolution.

My work demonstrates that ovariole number variation in *Drosophila* is an attractive trait for such an "eco-evo-devo" model. Ovariole number presents many interesting problems across different scales of biological organization, from molecules and cells (organization of TFCs into individual TF stacks) to populations and ecosystems (ovariole number evolution among the Hawaiian Drosophilids with respect to varying diet and habitat). Importantly, ovariole number in *Drosophila* is amenable to analysis on all of these scales. Although I have concentrated my efforts on IIS variation between two specific *Drosophila* species, there is still much to learn about the evolution of ovariole number. Next, I describe but a few interesting directions this research can take.

Future Directions

While this thesis presents significant advancement of our understanding of ovariole number determination and the mechanisms generating ovariole number diversity, interesting and important questions remain outstanding.

Potential Role of TF Stacking in Ovariole Number Evolution

Early ovariole formation presents an interesting model for a classic cell biology problem: How do groups of cells achieve a certain shape? Over the final 24 hours of larval development, TFPCs organize into a specific number of stacks, each of which contains the same number of cells. Thus, moreover, TF stack number determination addresses the poorly understood question of how counting is achieved amongst a group of cells (counting the number of stacks and the number of individual cells within a stack). Although counting occurs throughout multicellular life, for example specifying seven cervical vertebrae in most vertebrates, proximal molecular mechanisms are yet to be well characterized.

The molecular genetic mechanisms specifying a specific number of cells within a TF stack are largely unknown. Previous reports have shown that this parameter is under both environmental (Sarikaya et al., 2012) and genetic (Bartoletti et al., 2012; Bolívar et al., 2006; Hodin and Riddiford, 1998) control. In some lineages, TF cells per stack is significantly greater than the 7-9 cells per stack found in all of the lineages described within this thesis (Didem Sarikaya, personal communication).[1] I have observed that

[1] One example is *Drosophila teissieri*, a melanogaster subgroup species. A second example is a Hawaiian Drosophilid species. The Hawaiian Drosophilids have remarkably divergent ovariole number, ranging from 2-100. This divergence has evolved over a

TFPCs and TFCs within stacks express higher levels of the homomeric cadherins DE-cadherin (DE-cad) and DN-cadherin (DN-cad) compared to other somatic and germ cells of the ovary (Figure 5.1). Traffic jam, a transcription factor known to control TF morphogenesis, regulates expression levels of several adhesion molecules, including DE-cad, in the ovary (Li et al., 2003). It may be possible that modulation of DE-cad expression levels, mediated by traffic jam, may change stacking properties of TFPCs, leading to changes in TFC number per stack. Furthermore, in a lacZ enhancer trap screen, Godt and Spradling identified several putative regulators of TF morphogenesis as suggested by TF-specific lacZ expression (Godt et al., 1993; Godt and Laski, 1995; Ruohola et al., 1991). The identity of most of these genes is currently unknown. Finally, abrogation of ecdysone nuclear hormone signaling, via mutation of the nuclear receptors *Ecdysone Receptor* and *ultraspiracle*, disrupts both stacking and the timing of TFC stacking (Gancz et al., 2011; Hodin and Riddiford, 2000a). Therefore, one hypothesis for how different TFC numbers per stack is generated is that the dynamics of ecdysone signaling differ in different lineages, altering the number of cells competent to enter a stack or the duration of time that TFCs can form stacks. If TFC per stack number is increased, yet the size of the TFC pool is constant, TF number would be decreased, and vice versa. Identifying these genetic regulators and mechanisms of TF stacking may uncover additional ways by which evolutionary genetic change in ovariole number has occurred among Drosophilids.

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relatively short span of time (<7 million years) (Kambysellis and Heed, 1971). For comparison, wild type *D. melanogaster* and *D. sechellia*, which have mean ovariole numbers of 18.2 and 7.6, respectively, are approximately 5 million years diverged from a last common ancestor. It is tempting to conjecture, then, that even more diverse mechanisms may have been employed within the Hawaiian Drosophilids to vary ovariole number.

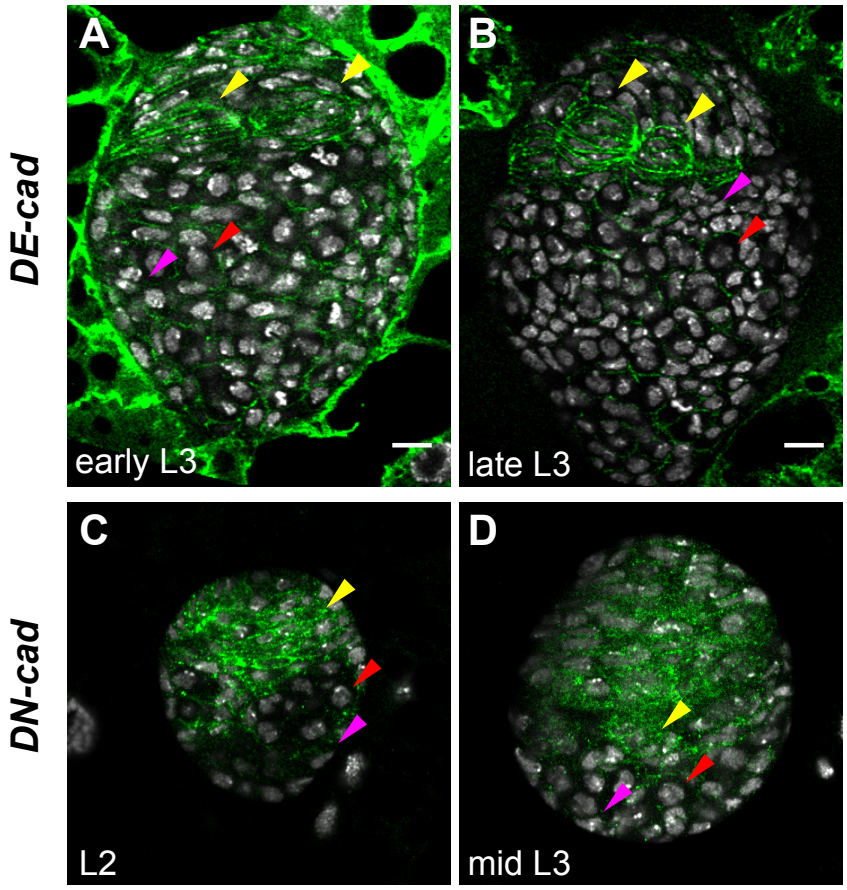

Figure 5.1: Expression of *DE-cadherin* and *DN-cadherin* in *D. melanogaster* larval ovaries. Images are confocal micrographs of a single z-plane. Larval stage is indicated in each panel. Cadherin expression is labeled in green. Rat anti-*DE-cad* and rat anti-*DN-cad* (used at 1:50) were obtained from the Developmental Studies Hybridoma Bank. Yellow arrows point to TFPCs or TFCs, depending on stage of ovary. Red arrows point to germ cells (identified by diffuse chromatin). Purple arrows point to posterior somatic cells. Orientation of panel (D) obscures clear stacking morphology, however stacking is occurring in this ovary. Staining conditions, image acquisition settings, and digital contrast settings are not constant across images. Scaling information was unavailable for DN-cadherin panels ((C) and (D)). Scale bar is 20 μm in (A) and (B).

Investigating the role of egg size and cell size in determining SGP number in Drosophila

We have much to learn about patterning and morphogenesis of the early ovary. One interesting problem is how the TFPC input pool is initially established. A surprising finding of my work was that SGP number established in embryogenesis differs between *Drosophila* species (Green and Extavour, 2012). Considering the genetic mechanisms that regulate the allocation of SGPs in the embryo, I developed a more specific model of how SGP number is determined in different species. This model is ultimately based upon cell number and size in the embryo, but also incorporates egg size, a trait that shows a positive correlation to egg hatchability (Azevedo et al., 1997), and thus to fitness.

Among other factors, expression of the transcription factor *engrailed* (*en*) specifies SGPs in the ventrolateral mesoderm of parasegments 10-12 in the *Drosophila* embryo (Riechmann et al., 1998). *en* is a segment-polarity gene that defines compartment boundaries within the tissues in which it is expressed, including specifying the anterior boundary of *Drosophila* embryonic parasegments. The resulting *en* expression pattern in the embryonic blastoderm is 14 circumferential stripes. In all cells in which it is expressed, *en* directs expression of *hedgehog* (*hh*), an intercellular peptide ligand. *en/hh* signaling is normally restricted to a very specific set of cells through an intercellular feedback mechanism involving a second segment-polarity gene *wingless* early in *Drosophila* embryonic development. Ectopic expansion and reduction of the number of cells expressing *en/hh* increases and decreases SGP number, respectively (Riechmann et al., 1998). It is plausible, then, that changing the number of cells within the *en/hh* domain may be a method of naturally varying SGP number.

Much work has attempted to describe how embryonic patterning is maintained

with respect to varying egg size. In a study of stripe scaling of *even-skipped*, an indirect regulator of *en*, in different-sized Drosophilid eggs, Lott et al. (Lott et al., 2007) showed that larger eggs contain more nuclei than do smaller ones. However, cell density (at early blastoderm stage) is lowered. This implies that larger eggs are composed of more, larger cells than smaller eggs. One of the well-known peculiar features of *D. sechellia* is its large egg size, 20% bigger than that of *D. melanogaster*. Lott et al. (2007) showed that *D. sechellia* has the lowest cell density, but largest egg size, of the species they consider (*D. melanogaster*, *D. sechellia*, and *D. simulans*). Chahda et al. (2013) reported that physical changes in nuclear size and packing can generate differences in the number of mesodermal cells specified across *Drosophila* (Chahda et al., 2013).

In Chapter 1, I showed that *D. sechellia* specifies fewer SGPs than does *D. melanogaster* (Green and Extavour, 2012). If the width of the *en/hh* stripe that specifies SGPs in *D. melanogaster* and *D. sechellia* is the same, or similar enough that a new row of mesoderm cells is not specified in *D. sechellia*, this would explain how larger *D. sechellia* cells could lead to fewer SGPs than in *D. melanogaster* (Figure 5.2). Furthermore, with respect to data I collected in an RNAi pilot screen (described in Appendix C), this would explain why increased IIS driven in the *hh*-expressing domain leads to decreased ovariole number and vice versa (Figure C.2). It would be interesting to determine the following:

1. If the width of *en/hh* stripes differs between *D. melanogaster* and *D. sechellia* in stage stage5/6 embryos, when *en* initiates its embryonic segment polarity expression pattern, and stage 10 embryos, when SGPs are distinct from fat body precursor cells but have not yet coalesced (Riechmann et al., 1998).

2. If cell number and size in stage 5/6 blastoderm embryos correlate with embryonic mesoderm cell number and size at stage 10.
3. If cell number and size in stage 5/6 blastoderm embryos contribute to SGP number.

It may also be interesting to investigate the molecular mechanisms that control differential egg size between different *Drosophila* species. It is likely the case that the IIS pathway plays a role here. Vitellogenesis, nutrient or yolk deposition in the oocyte, plays a primary role in oocyte growth and maturation. Vitellogenesis is regulated by hormonal signaling, including ecdysone signaling, juvenile hormone signaling, and IIS ((Barth et al., 2010; Richard et al., 2005); reviewed in (Swevers et al., 2005)). Nutrition also plays a strong role in controlling vitellogenesis, and this response is mediated through IIS (Barth et al., 2010).

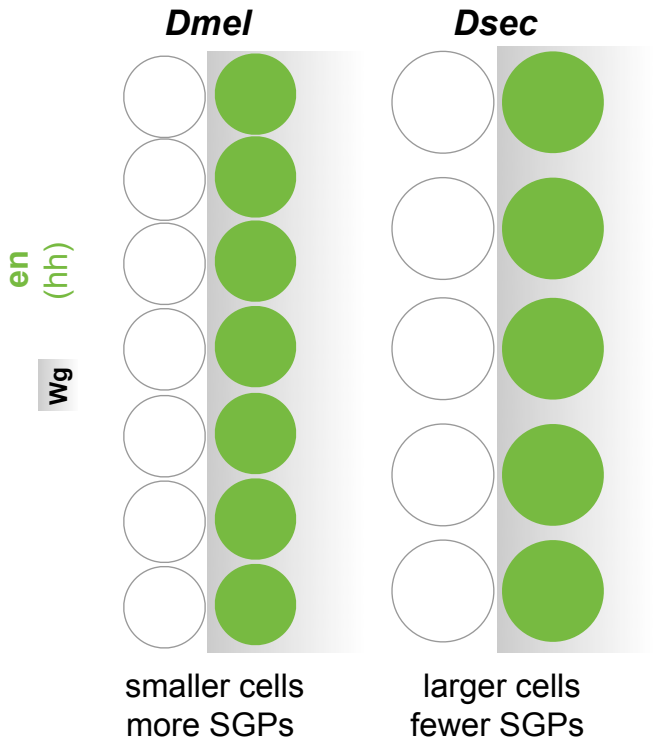

Figure 5.2: A hypothetical model for the effect of cell size on embryonic specification of SGPs. Feedback between hedgehog and wingless signaling creates sharp boundaries between the cells expressing either ligand. As a result, expression of *engrailed* (*en*) and *hedgehog* (*hh*) appear as circumferential stripes (green circles) along the *Drosophila* embryo (anterior is to the left). The extent of Wingless signaling controls the number of cells that will express *en* and *hh*. If the stripe width remains constant, one prediction is that smaller or larger cell size will result in greater or fewer cells expressing *en*/*hh*. The number of *en*/*hh*-expressing cells affects SGP number.

The finding that in *Drosophila* adult ovariole number is determined in pre-adult stages is significant for thinking about the evolution of life-history traits. Other insects similarly integrate environmental condition prior to reproductive maturity when establishing ovariole number. For example, differential late larval ovarian development in queen and worker honeybees is strongly influenced by nutrition, and is mediated through insulin/insulin-like growth factor (IIS) signaling (Ament et al., 2008). These findings suggest that insects have evolved reproductive bet-hedging strategies that are controlled by the environment, and that IIS controls this response. SGP specification and subsequent ovariole number determination presents an interesting model for studying how this transgenerational control of reproductive capacity is determined.

The role of development in generating and maintaining heritable variation in complex traits

My thesis focuses on more precisely defining changes in the function of a particular genetic locus that controls specific developmental mechanisms relevant to determining ovariole number. The assumption underlying my work was that knowing the developmental genetic details of ovariole number determination would contribute to narrowing the loci relevant to natural variation in ovariole number. While carrying out my developmental studies, however, I asked if our newly generated description of larval ovary development may be used in a different way to uncover the genetic basis of ovariole number evolution. This analysis would leverage a popular resource within the *Drosophila* community for the analysis of population genomics and quantitative traits: the *Drosophila melangoaster* Genetic Reference Panel (DGRP). The DGRP is a

collection of fully sequenced isofemale lines that has been created for the purpose of performing genome-wide association mapping studies (GWAS) in *Drosophila* (Mackay et al., 2012).[2] Use of the panel only requires measuring the phenotype of interest across the lines of the panel. An online tool is available[3] that provides statistical measure (*p* value) of likelihood of significant association for all SNPs across the genome.

In complex traits where distinct developmental and genetic mechanisms contribute to the final phenotype, for example *Drosophila* sex combs (Atallah et al., 2012; Kopp, 2011) and ovariole number (Green and Extavour, 2012; Hodin and Riddiford, 2000b; Sarikaya et al., 2012)), the potential evolutionary landscape may be broad. In these cases it is unclear to what extent different developmental mechanisms cause natural variation in the "final" trait among different lineages, and what this implies about evolution of the phenotype and of the organism. Are different developmental routes chosen at random to achieve the same end result? I believe my data would suggest that this is not true for ovariole number. In the case of *Drosophila* sex combs, it is clear that multiple transitions between the different modes of sex comb development have occurred between the species of the *melanogaster* and *obscura* species groups (Tanaka et al., 2009).[4] Tanaka et al. speculate that evolution of sexually dimorphic expression of the transcription factor *sex combs reduced* may control the switch in mode. One way to

[2] The DGRP is a collection consists of whole-genome sequence for 192 inbred isofemale lines derived from a wild population in North Carolina. Genome-wide SNPs have been identified and adult transcriptomes are available for a subset of 40 lines (Mackay et al., 2012).

[3] http://dgrp.gnets.ncsu.edu/

[4] Sex combs are unique to species within the *melanogaster* and *obscura* species groups. These two groups last shared a common ancestor 30-35 million years ago (Gao et al., 2007).

explore this problem systematically is to determine the extent to which genetic variation underlying differences in ovary development also contributes to ovariole number variation within a *D. melanogaster* population.

First, in order to obtain a higher resolution map of genetic variation underlying ovariole number variation, a GWAS may be performed for adult ovariole number.[5] Importantly, however, GWAS can also be performed on the individual developmental mechanisms that control ovariole number determination, including SGP number, TFC number, TFC number per stack, larval-pupal transition (LP) stage somatic cell proportioning, and larval ovary volume growth rate (Green and Extavour, 2012; Sarikaya et al., 2012). Each of these parameters is a quantitative trait and thus amenable to GWAS analysis. In Chapter 2, I showed that ovariole development proceeds differently in different populations of the same *Drosophila* species (*Oregon-R* and *India*), suggesting that segregating genetic variation exists within *D. melanogaster* that controls these different developmental processes (at least somatic cell proportioning, the principal developmental difference identified between these two lineages). This project would involve evaluating the following developmental parameters controlling adult ovariole number in all DGRP lines:

1. SGP number (count traffic jam$^+$ cells in L0 larvae)
2. LP stage TFC number (count En$^+$ cells in LP stage larvae)
3. LP stage TFC number per stack (count En$^+$ cells within a subset of stacks in LP stage larvae)
4. LP stage somatic cell proportioning (develop proxy from LP stage ovary images)

[5] I recently learned that this analysis has recently been completed by another group (via Cassandra Extavour, personal communication).

5. larval ovary growth rate (approximate growth rate by ovary volume increase using images of L0 and LP stage ovaries)

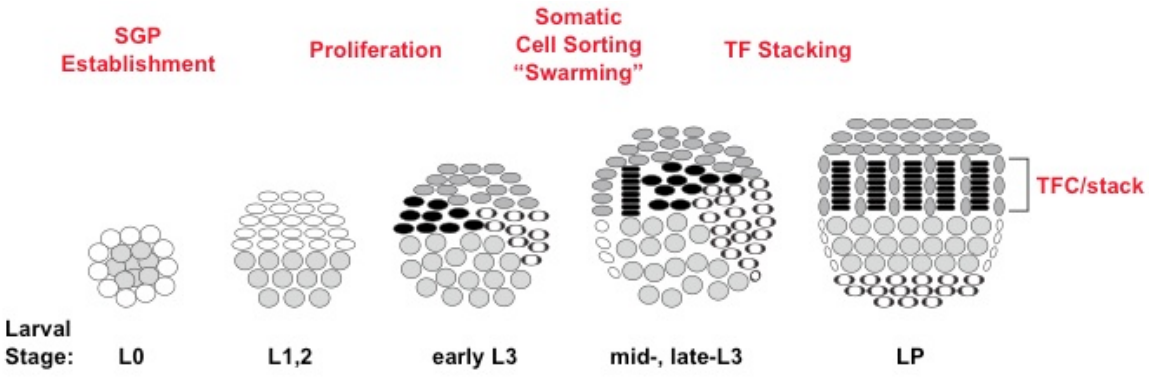

Figure 5.3: Schematic of ovary development and ovariole formation, including parameters identified to be relevant to ovariole number determination. *Drosophila* lineages differ in the number of SGPs they specify (parameter #1 from text). Somatic cells proliferate at different rates throughout larval life (parameter #5). A lineage-specific proportion of anterior somatic cells will migrate (a process called "swarming") to the posterior of the ovary (parameter #4), resulting in a specific number of anterior somatic cells from which TFCs will differentiate (parameter #2). Although not observed in the lineages studied here, the number of TFCs within a stack can differ between lineages (parameter #3), and thus is an important parameter in determining final TF number.

A list of SNPs with significant association would be generated for each phenotype. The resulting SNP association lists could be compared, and the following questions addressed:

- To what extent do SNPs associated with variation in developmental parameters overlap with SNPs associated with adult ovariole number?
- Do different developmental parameters contribute equally to ovariole number variation within this population?
- Is it possible/useful to refine candidate QTL lists by concentrating on overlapping SNPs?
- To what extent is variation in a complex trait (e.g. adult ovariole number) a reflection of variation in underlying development (vs other, indirect phenotypic variation)?

With this work, broader conclusions can be made about the role of development in generating and maintaining heritable variation in complex traits.

Conclusion

I agree with the assertion that the theory of genetic evolution will best be advanced by the addition of models that can be probed on a range of biological complexity. The *Drosophila* model system presents tremendous advantages for developing such models, including but not limited to a vast collection of genetic mutants, powerful and sophisticated genetic techniques, extensive genomic resources, and an abundance of data of its natural history and ecology. My goal with this work was to develop the *Drosophila* ovariole number model as a trait within the QTG program. My

initial impression was that as a reproductive trait with clear, albeit nontrivial, relationship to fitness, ovariole number may represent one of a few traits in which molecular genetic information might be directly incorporated into population genetic and ecological models. I recognize now the perhaps naïve ambition of such a goal. Nevertheless, my work does suggest that the *Drosophila* ovariole number model has the potential to contribute new insights into the QTG program. I believe an important insight drawn here, which has been summarized throughout this work and within this discussion, is the importance of considering trait development in studies of the genetic basis of phenotypic variation. Even without quantitative genetics studies to single-gene or single-mutation resolution, we were able to draw important conclusions about ovariole number evolution. Classical models of the genetics of adaptation (e.g. (Orr, 1998)) contain little formal treatment of development, despite their concern with such phenomena as pleiotropy and plasticity, which are developmentally based. This is likely a result of the relatively recent integration of molecular developmental genetics into evolutionary theory. I think this model highlights the utility of an understanding of developmental mechanism to studies of evolutionary genetics.

The *Drosophila* ovariole number model shares a challenge with most traits within the QTG program. One of the most prominent criticisms of the QTG program is whether or not ascertainment bias, or our ability due to technological and practical constraint to preferentially detect large-effect mutations versus small-effect mutations, invalidates the broad conclusions that have been and can be drawn from current examples within the "Loci of Repeated Evolution" (reviewed in (Rockman, 2011)). Are large-effect mutations accurately representative of the majority of evolutionarily relevant mutations? Classical

theory suggests that large-effect mutations should represent a small fraction of evolutionarily relevant mutation (historical overview in (Orr, 2005)). Indeed, in this work I do focus on a locus of strong effect from an interspecies QTL study among the many loci found to control ovariole number variation (Orgogozo, 2006). I believe that this criticism is fair. Nevertheless, new data are emerging to suggest a biological, versus technological, explanation for the predominance of large-effect loci within the "Loci of Repeated Evolution". In a number of cases, large effect loci are composed of multiple tightly linked moderate- to small-effect mutations, each of which has limited pleiotropic effects (Linnen et al., 2013; Martin and Orgogozo, 2013). I do not have sufficient resolution with my own work to suggest that this is the case with respect to ovariole number variation. Despite the potential limitations of conclusions that can be made from the collection of genetic variants in the "Loci of Evolution," as Rockman (2011) points out, each case within the QTG program does stand on its own. As the *Drosophila* ovariole number model is expanded, in this lab and others, I am eager to see what additional insight it may provide.

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Appendix A

Counting in Oogenesis

Abstract

The determination of a precise number of cells within a structure and of a precise number of cellular structures within an organ is critical for correct development in animals and plants. However, relatively little is known about the molecular mechanisms that ensure that these numbers are achieved. We discuss counting mechanisms that operate during ovarian development and oogenesis.

Introduction

The study of the molecular genetic control of animal development has made great progress in two major areas: differentiation and proliferation. In many cases, a circuit-like network that regulates gene expression controls differentiation. Input to the circuit can be in the form of signals exchanged between cells or of a transfer of information through an intracellular cascade. The ultimate goal is to ensure that the expression of genes dictating different cell fates is achieved in the appropriate cells and is prevented in others. Differentiation is not simply a binary switch: the control of the number of cells that acquire a specific fate is also a part of the differentiation process.

Great strides have also been made toward understanding proliferation, although here the problem is more complex. Whereas several mutations have been discovered that result in over- or under-proliferation of cell populations, the precise control of tissue-specific proliferation parameters is less well understood. Proliferation can be regulated either by non-autonomous control of cell cycle switches or by intrinsic control of a certain number of divisions and the molecular mechanisms involved can be markedly different in each case. This paper will deal with a third major problem, the problem of

counting during development; this issue is at the interface of both differentiation and proliferation. Here, we use "counting" to refer to developmental decisions whereby specific numbers of groups of cells must adopt a certain fate or undergo a collective morphogenetic process to form a single structure.

The development of the reproductive system and the process of gametogenesis provide several clear instances of the precise genetic control of counting. Because the molecular genetic mechanisms of these processes are best understood in *Drosophila*, we will focus on two major instances of counting necessary for *Drosophila* oogenesis: the number of germ-line stem cells (GSCs) that undergo divisions and the number of structures that house the GSCs. Following an introduction to the structure of the *Drosophila* ovary, we will discuss examples of recent advances in understanding the method of counting in the GSC niche and conclude with the comparatively unexplored area of the genetic control of ovariole number determination.

Structure of the fly ovary

In all insects, each of the paired ovaries (Figure A.1A) is partitioned into functional units called ovarioles. These act as assembly lines in which oogenesis proceeds, with progressively older oocytes being arranged from anterior to posterior (Figure A.1B). The anterior tip of each ovariole consists in a stack of somatic cells called terminal filament (TF) cells (Figure A.1C). Immediately posterior to the TF is the germarium where the process of oogenesis begins. The germarium contains GSCs, somatic stem cells, cap cells, gonia and early cysts, which ultimately give rise to the oocyte. Newly eclosed adult females possess two to three GSCs (Figure A.1C) tethered to

somatic cells called cap cells, which secrete signals that maintain the stem cell population and are part of the stem cell niche. GSCs undergo asymmetric division, giving rise to one daughter cell that remains attached to the cap cells and another that is not in contact with the cap cells. The former cell remains a GSC by virtue of its contact with the niche, whereas the latter cell proceeds to gametogenesis. The oogenesis developmental program begins with four mitotic divisions called transit-amplifying (TA) divisions, whose products are surrounded by follicle cells, the daughters of somatic stem cells. Together, the 16 clonally related germ cells and their encapsulating follicle cells are called a cyst. Of the 15 cyst cells (called cystocytes) undergo rounds of endoreduplication and become polyploid nurse cells, which will contribute to oogenesis by providing the 16th cell, the future oocyte, with the mRNAs and proteins necessary for early embryonic patterning. All cells of a single cyst thus ultimately produce a single oocyte.

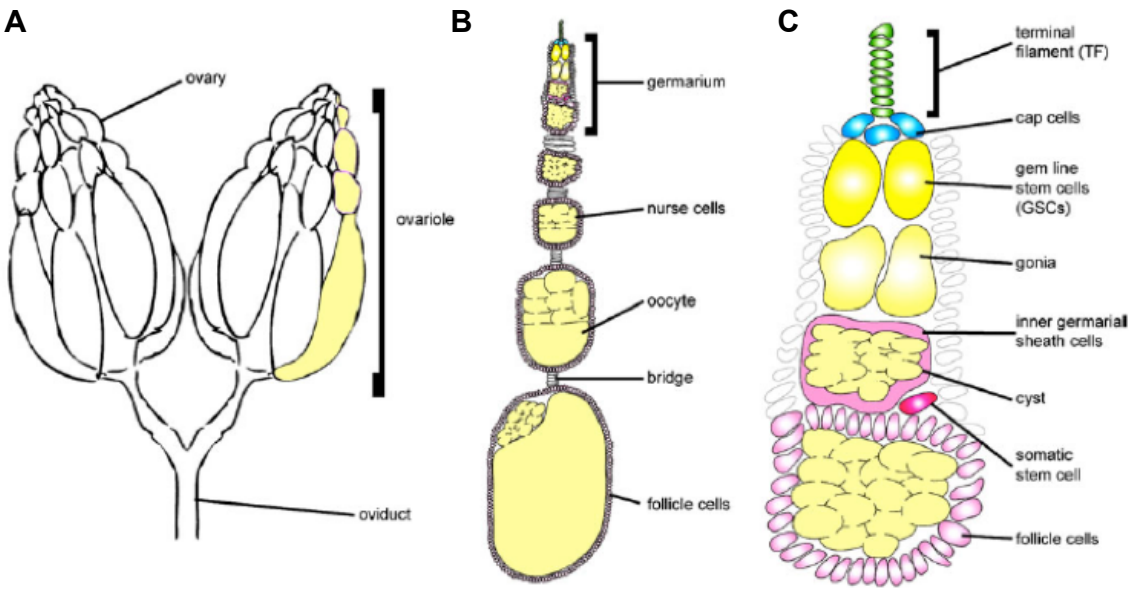

Figure A.1: Anatomy of an adult female ovary and oogenesis in Drosophilids. (A) Each of the two ovaries has individual oviducts that are connected by a common oviduct at the posterior. Each ovary is composed of several ovarioles. (B) Individual ovariole from a Drosophilid ovary. The germarium is at the anterior, followed by egg chambers at successively older stages of development. Egg chambers consist in one oocyte (at the posterior) and 15 interconnected nurse cells (at the anterior). Each egg chamber is surrounded by a complement of follicle cells and connected by follicular bridge cells. (C) Germarium of an adult female ovary. Oogenesis begins at the anterior tip of the gonad (green terminal filament cells, blue cap cells, dark yellow germ-line stem cells [GSCs], light yellow gonia and differentiating gametogenic cells [cysts], dark pink follicle [somatic] stem cells, light pink follicle cells). Anterior is up.

The next section examines the counting mechanisms controlling the differentiation and proliferation of the cells that produce differentiating gonia: the GSCs.

Counting GSCs

Oogenesis requires the regulation of the counting of two critical parameters: the number of GSC divisions and the number of GSCs themselves. The number of divisions that each GSC undergoes determines the number of eggs produced by the female and the reduction in number of GSC divisions over the animal's lifetime is responsible for decreased fecundity with age (Zhao et al., 2008). Compared with 3-day-old females, the GSC division rate is reduced to 50% by 15 days and to 25% by day 40. This has a direct effect on fecundity, as egg production in these flies is reduced to 50% by 15 days and almost no eggs are produced by day 40. Flies carrying a mutation in the *Drosophila insulin receptor* homolog also have a reduced GSC division rate, suggesting that the insulin pathway and
nutrition have an effect on the counting of GSC divisions (H.-J. Hsu and Drummond-Barbosa, 2009).

Similarly, both insulin signaling and aging also influence the number of GSCs in the germarium (LaFever, 2005; Zhao et al., 2008). The average GSC number per germarium decreases from 2–3 in young flies to 0–2 in old flies (Zhao et al., 2008). Interestingly, the counting of GSCs is closely tied to the interaction of these
cells with the cap cells. When cap cells are induced to upregulate bone morphogenetic protein signaling, which is required for the maintenance of GSCs, older flies retain more GSCs than wild-type flies of the same age (Zhao et al., 2008). However, these flies only

have a higher egg production rate as young adults, suggesting that fecundity is not simply a function of the absolute number of GSCs. Young flies with defective insulin signaling also show reduced GSC numbers, as reported by Hsu and Drummond-Barbosa (2009); although the authors have not reported the fecundity of these flies, the reduction of the stem cell population implies the cessation of de novo gametogenesis in those ovarioles, leading thereby to a reduction in fecundity. Whereas the factors determining their functionality are likely complex, the number of GSCs is clearly tightly regulated and the cap cell population appears to influence this instance of counting in the germarium. Given the central role of cap cells in the counting process, an understanding of the developmental origin, maintenance and roles of these cells is of important.

Cap cells are located posterior to the TF cells and anterior to the germ cells (Figure A.1C). Newly eclosed adults have four to five cap cells per niche. The role of cap cells in maintaining the appropriate numbers of GSCs has been illustrated by Hsu and Drummond-Barbosa (2009) who have found that, similar to the GSC number, the cap cell number declines as flies age. Moreover, flies that are mutant for the insulin receptor form fewer cap cells, which are lost more readily than in wild-type (Hsu and Drummond-Barbosa 2009). These flies then go on to lose GSCs because of insufficient signals from the cap cells. The counting of the cap cells is thus critical for the proper establishment and maintenance of the correct number of GSCs.

Cap cells originate from somatic cells adjacent to TF cells in the larval ovary, in a process that Song and colleagues (Song et al., 2007) have shown takes place at the larval-pupal transition and involves Notch signaling. When Notch signaling is ectopically activated in somatic cells surrounding GSCs, ectopic cap cells form by the recruitment of

inner germarial sheath cells to a cap cell fate (Song et al., 2007). Similarly, the cap cell number also increases when GSCs are induced to overexpress the Notch ligand Delta (Ward et al., 2006). Not only do these flies have almost three times as many cap cells as young wild-type adults, the cap cell number also increases throughout adulthood. Taken together, these observations suggest that during development, many somatic cells surrounding GSCs are competent to differentiate into cap cells via Notch signaling but this differentiation normally takes place only in the four to five cells that are immediately anterior to germ cells in each ovariole.

Because the activation of canonical Notch signaling requires the Notch receptor to interact with its membrane-bound ligand, the receptor-expressing and ligand-expressing cells must be in physical contact with each other. During the normal establishment and maintenance of cap cells, it is therefore important to know the location of the expression of Notch and its receptors. At the larval-pupal transition, two cell types express Delta: GSCs and TF cells. Ward and colleagues (2006) have reported that Delta mutant GSCs are lost from the niche, suggesting that Delta expression plays a role in maintaining GSCs in the niche. However, in a subsequent study, Hsu and Drummond-Barbosa (H.-J. Hsu and Drummond-Barbosa, 2011) have observed neither a difference in GSC maintenance in niches containing Delta mutant GSCs compared with wild-type niches, nor a change in the cap cell number. These authors conclude that Notch ligands expressed in the GSCs do not play a role in cap cell counting. However, flies that do not have germ cells can form germaria without cap cells (Song et al., 2007).

TF cells also express Delta (Song et al., 2007). There are seven to ten TF cells per TF and TF cells can affect the cap cell number when the TF cell directly in contact with

the cap cells lacks Delta activity (H.-J. Hsu and Drummond-Barbosa, 2011). Whereas the cap cell number is lower in these individuals, the number does not change significantly in adulthood, suggesting that Delta signaling from the TF plays a role during the organization of the organ during larval and pupal development but not in its maintenance during adulthood.

Currently, at least two important aspects of cap cell counting remain to be established: (1) the role of GSCs in cap cell formation and (2) the role of Delta signaling within cap cells. The current state of knowledge does not allow us to distinguish between a model in which the cap cells and GSCs regulate each other's numbers homeostatically (as has been observed for germ cells and intermingled cells in the larval ovary; (Gilboa and Lehmann, 2006)) and one in which the correct number of cells is first established in one of these cell populations and subsequently determines the number of cells in the other population. To our knowledge, no quantitative reports are available on how the loss of GSCs affects the cap cell number; this would be an interesting topic for future investigations and would help elucidate the role of signals from the GSC.

With respect to the second issue, the signaling via Delta is clearly important for cap cell number determination and maintenance but topics that remain to be resolved include the identification of the sources of the relevant signals and whether these sources are the same during ovarian development and throughout adult life. In larval and early pupal stages, Delta expression has been reported only in TF cells and GSCs (Song et al., 2007), suggesting that these cells induce competent somatic cells to become cap cells. However, the observations that adult cap cells are established and maintained in niches (1) with compromised GSC Delta function, (2) with compromised Delta function in the

TF cell in contact with cap cells (H.-J. Hsu and Drummond-Barbosa, 2011), or (3) that lack GSCs entirely (Y.-C. Hsu et al., 2007) suggest that a non-GSC non-TF source of Delta might operate in adult niches. On finding that cap cells in normal adults express Delta and that some cap cells contact only each other and not TF cells or GSCs, Hsu and Drummond Barbosa (H.-J. Hsu and Drummond-Barbosa, 2011) have proposed that Delta expression within the cap cell population provides sufficient signaling to establish and maintain correct cap cell numbers. To test this hypothesis, the determination of whether Delta expression can be detected in cap cell precursors or in cells adopting the cap cell fate during mid to late pupal stages would be of interest. Finally, although Notch signaling is the only pathway that has been specifically implicated in this process to date, additional signals might play a role in cap cell differentiation.

Counting during ovarian morphogenesis

Counting is integral for the precise construction of the gonad in the hexapods (Figure A.1A). Ovariole count is variable among insect species, ranging from one (in a Hawaiian fruit fly) to 1000 (in a beetle) per ovary (Büning, 1994; Kambysellis and Heed, 1971). However, the number is specific within species. For example, adult females of wildtype (Oregon R) Drosophila melanogaster have 18±2 ovarioles at 25°C. The rate of egg production is constant per ovariole (maximum 2 eggs/ovariole per day in D. melanogaster) and independent of the number of ovarioles present in a single ovary (Cohet and David, 1978). Egg production rate is positively correlated with the ovariole number, making it a strong determinant of reproductive capacity and hence of fitness (Cohet and David, 1978).

The ovariole number is determined both environmentally and genetically. The majority of studies on this topic to date have taken ecological, evolutionary, or quantitative genetic approaches. Studies of laboratory populations have revealed that an intraspecific maximum ovariole number is attained with optimal larval nutrition at intermediate temperatures (Delpuech et al., 1995; Thomas-Orillard and Jeune, 1985). In studies of natural populations of *D. melanogaster* and *D. simulans*, the ovariole number has been found to vary along a latitudinal cline, distinguishing populations within each species (Capy et al., 1994; 1993; Gibert et al., 2004). Both species show a similar clinal variation in the ovariole number, establishing that different species respond in similar ways to environmental influences. This suggests a common adaptive genetic basis of the trait. The best-studied Drosophilid example of ecological differences in the ovariole number is that of *D. sechellia*. This species occupies a specialized ecological niche in the Seychelles and possesses half the number of ovarioles as the generalist *D. melanogaster* (Louis and David, 1986). The corresponding relative reduction in fecundity in *D. sechelli*a (R'kha et al., 1997) might be offset by the unique advantage it holds in colonizing its niche: it has evolved the ability to metabolize a toxin produced by its host fruit, which is lethal to the competitor Drosophilid species (R'kha et al., 1991).

Quantitative genetic analyses of recombinant inbred and mutation accumulation lines of *D. melanogaster* have demonstrated significant segregating variation for the trait and have identified several autosomal effect loci (Wayne et al., 2001; 1997; Wayne and McIntyre, 2002). Similarly, quantitative genetics approaches comparing *Drosophila* species (Coyne et al., 1991; Orgogozo, 2006) have validated older studies based on the coarse mapping of interspecies crosses (Thomas-Orillard, 1976) and indicate that the

principal loci that control number are found on chromosomes 2R and 3. However, specific genetic factors and an explanatory molecular genetic mechanism of counting have yet to be determined.

Consideration of ovarian development might shed new light on the ovariole number counting mechanism. In *D. melanogaster*, ovariole formation occurs in late larval life, beginning with the transformation of a group of anterior somatic cells into a specific number of organized stacks, the TFs (see above). Mutations that affect proper TF cell intercalation and recruitment (described in genes including *bric-á-brac*, *engrailed*, *hedgehog* and *twinstar*) lead to grossly abnormal ovarian morphology and adult female sterility, indicating that morphogenesis indirectly affects the ovariole number (Besse et al., 2005; Bolívar et al., 2006; Chen et al., 2001; Godt and Laski, 1995). Counting has been addressed more specifically in the analysis of mutations in the *ecdysone receptor* and *ultraspiracle* genes, which encode nuclear co-receptors that regulate metamorphosis. Mutations in these genes cause mild defects in TF morphology and reduced ovariole numbers but adult females are nonetheless fertile (Hodin and Riddiford, 1998). TFs in these mutants are composed of more cells and form later in development than those in the wildtype, suggesting that ovariole counting is mediated by the ecdysone-dependent temporal control of TF cell morphogenesis.

In a final instance of counting within the ovary, each TF stack is composed of 7–10 cells (Godt and Laski, 1995). The function and mechanism of this case of counting are unknown and largely unexplored. One hypothesis is that this specific cell number plays a structural role in early ovariole formation, as TF stacks serve as tracts along which apical cells travel and ensheath a pool of germ cells, thus delineating individual ovarioles.

Alternatively, a specific TF cell number might be involved in maintaining the GSC niche, as TFs lie adjacent to the cap cells that form the GSC niche and express similar signaling factors. The genes that affect TF morphogenesis are good candidates for regulators of this counting mechanism.

Concluding remarks

Our understanding of the way that counting is regulated at molecular and developmental levels during gonadogenesis and gametogenesis is not yet complete. Even the few examples discussed here raise several specific questions that remain to be answered in future work. For example, what are the downstream targets of Notch signaling that induce cap cell fate? What are the targets of evolutionary change that result in the species-specific ovariole number? Given that little is known about the mechanistic regulation of this process, interspecies comparisons that reveal genes that have changed during evolution to cause changes in ovariole numbers between species might be fruitful starting points to identify candidates for advanced molecular genetic analysis in *D. melanogaster*. Finally, the number of ovarioles that have GSCs, the number of GSCs and their division rate and the number of TA divisions undergone by the gonial cells produced by GSCs are parameters that must be integrated during development. How are these decisions coordinated during development and throughout reproductive life? Further work on the molecular genetic basis of these processes is needed to provide answers to these questions.

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Appendix B

Assessing the Effects of Modulating Ovary-Specific Insulin Signaling on Ovariole Number Determination: an RNAi Knockdown Pilot Screen

Introduction

Early in my work, I sought to test if IIS plays a role in ovary development, and that manipulating cell number and size specifically in the ovary leads to changes in adult ovariole number. Previous studies have shown that adult ovariole number is altered in *D. melanogaster* that harbor mutations in IIS pathway components. *chico*^{1} mutants have approximately half the number of ovarioles as their heterozygote siblings (Richard et al., 2005; Tu and Tatar, 2003). At the time of the pilot, ovariole number reduction was also reported as "data not shown" for a transheterozygous combination of *InR* loss of function alleles (Gancz and Gilboa, 2013; Green and Extavour, 2014; 2012; Tu and Tatar, 2003). In all of these cases, not only was ovariole number changed, but also body size, leaving open the question of the specificity and autonomy of the phenotype to somatic ovarian cells.

I chose to perform an RNAi expression knockdown pilot screen of IIS pathway components in order to address this specificity issue. I initially decided to use a *hedgehog*-GAL4 driver (*hh*-GAL4), as *hh* is expressed exclusively in terminal filament precursor cells (TFPCs) and remains expressed in TFCs once stacking is complete (Besse et al., 2002). I confirmed that this driver is indeed expressed exclusively within TFPCs and TFCs in larval ovaries beginning in the second instar (Figure B.1). I sought to extend my analysis and confirm any potentially significant differences with a second driver, *bric-á-brac*-GAL4 (*bab*-GAL4). *bab2*, the specific expression pattern reported by this driver, is expressed most strongly in TFPCs beginning in the second larval instar and in TFCs post-stacking, but also weakly expressed in other somatic ovarian cells (Figure B.1)

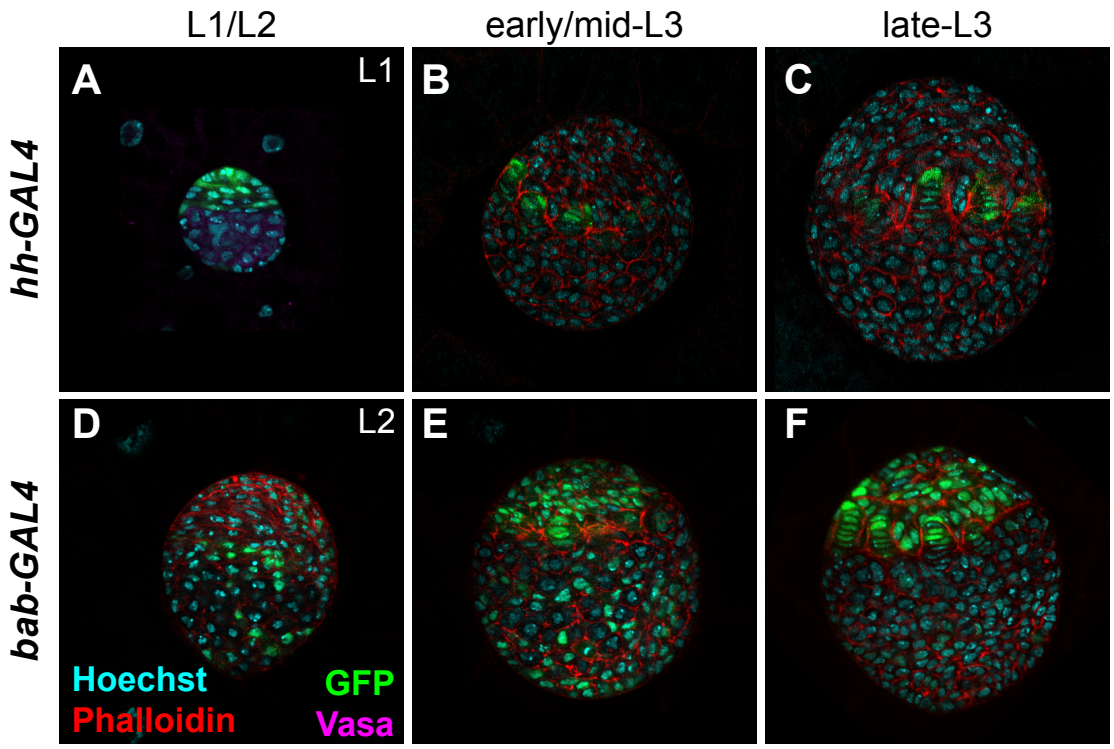

Figure B.1: GAL4 driver expression patterns in larval ovaries. Images are confocal optical sections of *D. melanogaster* larval ovaries at the indicated larval stages. GAL4 driver lines were crossed to a UAS-GFP reporter line. *hh*-GAL4 shown in top row (A-C), *bab*-GAL4 shown in bottom row (D-F). Nuclei are labeled in cyan. Cell membranes are outlined in red. Cells expressing the GAL4 driver are labeled in green. Germ cells are labeled in magenta in (A). (A) was obtained in a different experiment from other ovaries, hence it is the only ovary for which vasa was immunostained.

(Couderc et al., 2002; Godt et al., 1993; Godt and Laski, 1995).[1] I confirmed this expression pattern in this driver (Figure B.1).

Materials and Methods

Drosophila *lines* and *culture conditions*

All adult ovariole counts were performed as previously described (Sarikaya et al., 2012). Flies were maintained on standard lab diet (32g Torula yeast, 60.5g corn meal, 128g dextrose, 9.2g agar per liter). All rearing and experiments were performed at 25°C at 60-70% humidity.

To determine the responsiveness of somatic ovarian cells to IIS, we altered expression of the indicated IIS components via their respective *UAS* lines (corresponding Bloomington *Drosophila* Stock Center number in parentheses): *UAS-InR*^{RNAi} (BDSC #31037), *UAS-chico*^{RNAi} (BDSC #28329), *UAS-dilp2*^{RNAi} (BDSC #31068), *UAS-dilp3*^{RNAi} (BDSC #31492), *UAS-dilp4*^{RNAi} (BDSC #31377), *UAS-dilp6*^{RNAi} (BDSC #31379), *UAS-dilp7*^{RNAi} (BDSC #31069), *UAS-pten*^{RNAi} (BDSC #25841), *UAS-pten*^{RNAi} (BDSC #25967), *UAS-pi3k92e*^{RNAi} (BDSC #27690), *UAS-InR*^{K1409A} (BDSC #8259), and UAS-*InR*^{Exel} (BDSC #8262). RNAi lines were kindly provided by Norbert Perrimon (Harvard Medical School). All *UAS* lines were homozygous for the *UAS* construct on chromosome III except for *UAS-pten*^{RNAi} (BDSC #25967), which is balanced over the TM3, Sb[1] third chromosome balancer. *UAS* lines were crossed to *hh*-GAL4 and *bab*-GAL4 lines, which were both balanced over third chromosome balancers (*hh*-GAL4/TM2 and *bab*-GAL4/TM6B Tb[1]). Controls for homozygous lines were sisters carrying a balancer

[1] *Bab* is also strongly expressed in a small number of posterior somatic ovarian cells called swarm cells in late third larval instar.

chromosome. Controls for the *UAS-pten*^{RNAi} (BDSC #25967) cross were the *UAS-pten*^{RNAi}/(TM2 or TM6B Tb^{1}) and *hh*-GAL4/TM3 Sb^{1}. The *hh*-GAL4 line was a gift of Laura Johnston (NYU). The *bab*-GAL4 line was obtained from the BDSC (BDSC #6803) (Cabrera et al., 2002).

Student's *t* test was used for all pairwise comparisons of differences in means unless otherwise noted.

Results

From the loss-of-function mutant phenotypes in IIS components, I hypothesized that IIS promotes somatic ovarian cell proliferation and growth, and hence increases ovariole number. I expected knockdown of "positive" pathway components, including *InR*, *chico*, *pi3k92E*, and the *dilps* to decrease ovariole number and vice versa.[2] Unexpectedly, in the *hedgehog*-GAL4 (*hh*-GAL4) screen, RNAi against *InR* and *dilp3* resulted in 19.9% ($p<0.001$) and 18.0% ($p<0.001$) increases, respectively, in adult ovariole number compared to controls (Figure B.2). Consistent with these results, overexpression of wild type *InR* and a dominant negative *InR* allele (*InR*^{K1409A}, kinase "dead") resulted in adult ovariole number decrease (-10.4%, $p<0.005$) and increase (11.0%, $p<0.001$), respectively (Figure B.2). Finally, also consistent with this result was a significant reduction (-13.9%; $p<0.001$) in ovariole number with RNAi against *pten*,

[2] The pilot screen data do not include all major components of the IIS pathway. Two notable omissions include *akt* and *foxo*. These omissions were due to experimental design, specifically proceeding through the lines in phases and "blinding" lines to reduce bias. Given the large number of crosses, I planned to conduct the screen in phases. Because I blinded the lines to be screened, I did not know which lines would be analyzed within the first phase. As is described within the text, the experiment was eventually tabled due to confounding results, and thus specific components went untested.

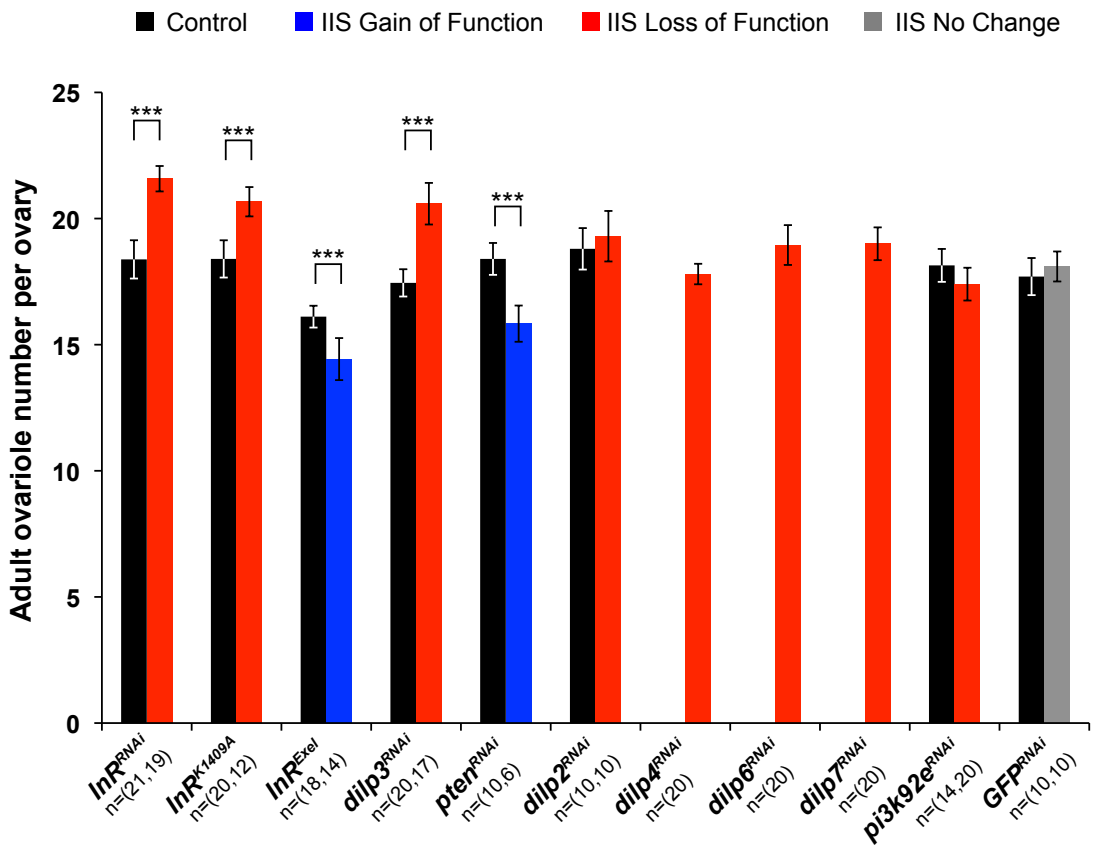

Figure B.2: Adult ovariole number in *hh*-GAL4 crosses. Control ovariole numbers (explanation in Materials and Methods section) are indicated by black bars. Experimental ovariole numbers are indicated by colored bars: expected gain of IIS activity (blue bars), expected loss of IIS activity (red bars), and no expected change in IIS activity (grey bar). Significant differences are indicated by brackets and asterisks. Error bars show 95% confidence interval. *** $p < 0.001$. *n* indicates number of ovaries analyzed for (controls,experimental).

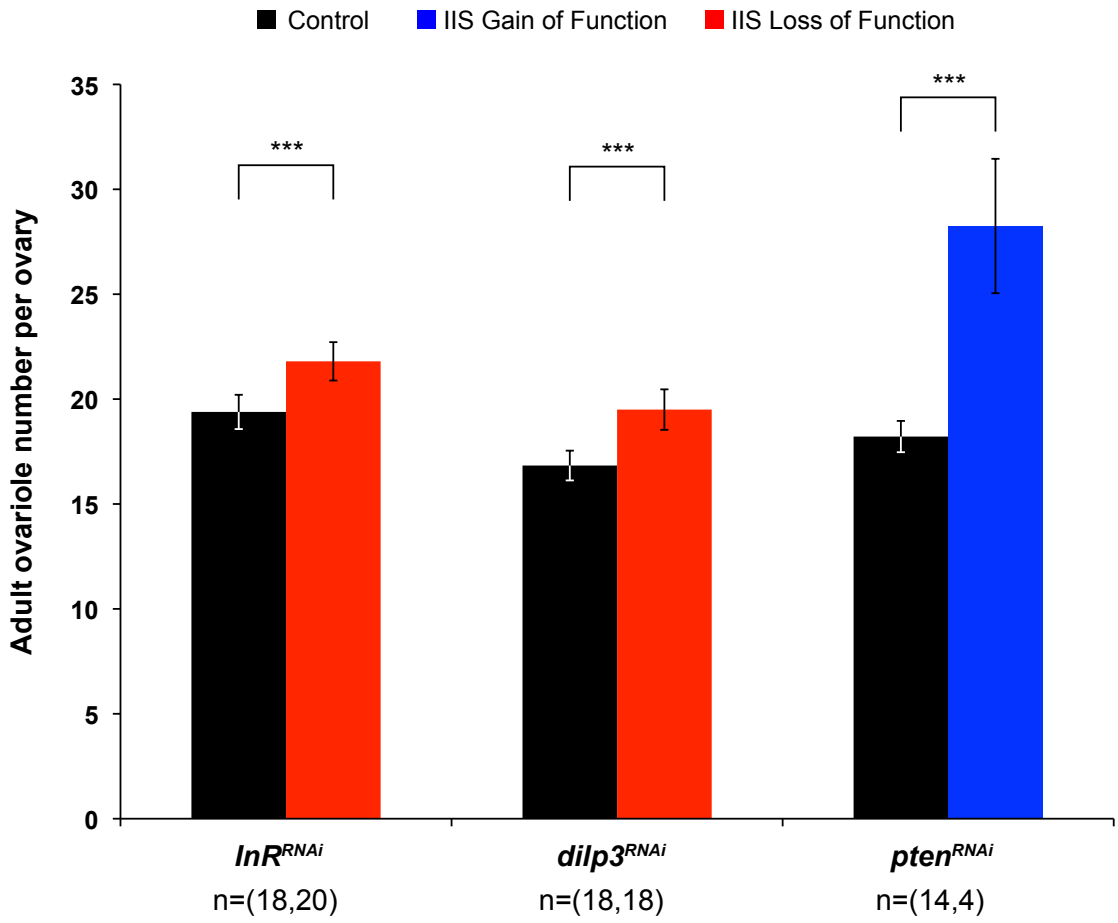

Figure B.3: Adult ovariole number in *bab*-GAL4 crosses. Control ovariole numbers (explanation in Materials and Methods section) are indicated by black bars. Experimental ovariole numbers are indicated by colored bars: expected gain of IIS activity (blue bars), expected loss of IIS activity (red bars). Significant differences are indicated by brackets and asterisks. *** $p < 0.001$. Error bars show 95% confidence interval. *n* indicates number of ovaries analyzed for (controls,experimental).

which is a negative regulator of IIS (Figure B.2). RNAi against *chico* was lethal. RNAi against *pi3k92E* and the additional *dilps* did not significantly change ovariole number compared to controls. I verified that results were not due to nonspecific effects of the balancer chromosome by testing a line generating RNAi against GFP, which should have no effect on adult ovariole number. No significant difference in ovariole number was observed (p=0.58) (Figure B.2).

I used the *bab*-GAL4 driver as one way to validate significant differences found with *hh*-GAL4. The *bab*-GAL4 driver showed similar results as with *hh*-GAL4. RNAi against *InR* and *dilp3* resulted in 12.4% (p<0.001) and 15.8% (p<0.001) increases, respectively, in adult ovariole number compared to controls (Figure B.3). One significant exception was that RNAi against *pten* resulted in massive increases (55% in B25841 and 108% in B25967; p<0.001 in both cases) in ovariole number in both *pten* RNAi lines (Figure B.3). This is a substantial difference compared to the 12-20% differences from controls observed with all other phenotypes. This suggests that the difference in ovariole number caused by changing *pten* expression with *bab*-GAL4 is happening in a quite different way than differences caused by manipulating other IIS components.

Discussion

This pilot screen was initially tabled and ultimately abandoned in its described form due to these confounding results. I returned to the more specific experiment of modulating *InR* expression levels in somatic ovarian cells with a more specific GAL4 driver. These results are described in Chapter 3. We, and others, show that changing expression levels of *InR* specifically in somatic ovarian cells is sufficient to change

ovariole number (Gancz and Gilboa, 2013; Green and Extavour, 2014), demonstrating that ovariole number difference is not simply a reflection of body size change, which is also altered in whole body mutants.. Increasing *InR* transcript levels promotes somatic ovarian cell proliferation and vice versa, confirming our original hypothesis that IIS indeed promotes proliferation. These experiments were performed with the the *c587*-GAL4 driver, which is expressed specifically in somatic ovarian cells beginning in the third larval instar (Manseau et al., 1997). These results raise an important question about how to interpret the data obtained from the pilot screen.

In an attempt to develop a biological explanation for these results, I reconsidered the genetic mechanisms that regulate the allocation of SGPs in the embryo. I developed a more specific model of how SGP number is determined in different species based upon embryonic cell number, cell size, and egg size. Discussion of this model is presented in Chapter 5 ("*Investigating the role of egg size and cell size in determining SGP number in* Drosophila"). In summary, modulation of IIS activity via the *hh*-GAL4 driver may have changed somatic gonad precursor (SGP) number through changing embryonic cell size. In addition to being expressed in TFPCs and TFCs, *hh* is also expressed in the embryonic mesoderm, which contributes to SGP number determination. I previously showed that SGP number is one developmental mechanism used to change ovariole number in *Drosophila* (Green and Extavour, 2012). This hypothesis does not explain, however, why decreased IIS activity via the *bab*-GAL4 driver should result in increased ovariole number.

It is also possible that these results reflect technical difficulties and shortcomings of the pilot study. All UAS lines used in the pilot were first-generation

VALIUM1/VALIUM10 constructs that used the long double-stranded hairpin approach (Ni et al., 2009; 2008), which have subsequently improved in the newer VALIUM20/VALIUM22 constructs, which use short hairpin microRNA technology (Ni et al., 2011). Thus it may be the case that lines for which no phenotype was observed was due to no or insufficient knockdown of target gene expression levels.

The results of this screen notwithstanding, it is clear that IIS activity plays a major role in controlling somatic ovarian cell proliferation, and hence ovariole number determination.

Acknowledgements

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Appendix C

Allele-Specific Expression of *InR* in *D. melanogaster* and *D. sechellia* Hybrids

Introduction

In Chapter 3 I presented results from interspecies hybrid complementation tests to test for species-specific differences in IIS-mediated control of TF number between *D. melanogaster* and *D. sechellia*. Data were consistent with the hypothesis that the wild type *D. melanogaster InR* allele confers a higher level of insulin/insulin-like growth factor signaling (IIS) than the wild type *D. sechellia* allele, furthermore consistent with IIS activity being higher in *D. melanogaster* compared to *D. sechellia* (Green and Extavour, 2014). Both coding (InR^{339} is a non-synonymous coding point mutation) and non-coding (InR^{GC25} is an inversion mutation within the putative *InR* upstream regulatory sequence that removes portions of the 5'UTR; *Df(3R)6186* is a deficiency that deletes portions of the 5'UTR) mutations show terminal filament (TF) number reduction phenotypes in hybrids between *D. melanogaster* and *D. sechellia* (Green and Extavour, 2014). This left unresolved the question of whether cis-regulatory or coding mutation controls interspecies divergence.

I sought to test the hypothesis that relative allele-specific, and thus species-specific, *InR* transcript expression level contributes to the TF number reduction phenotypes in hybrids. I began with two naïve assumptions. The first is that the intermediate TF number observed in wild type hybrids is due to a total *InR* expression level that is intermediate to that of the pure species of *D. melanogaster* and *D. sechellia*. The second is that the *D. melanogaster InR* allele confers greater expression of the *D. melanogaster InR* transcript relative to the expression level of the *D. sechellia InR* transcript driven by the *D. sechellia* allele. This would be consistent with the *D. melanogaster* allele conferring higher IIS activity compared to the *D. sechellia* allele.

Materials and Methods

Drosophila *strains* and *culture conditions*

The following strains were used as wild type strains: *D. melanogaster* Oregon R-C (Bloomington Drosophila Stock Center (BDSC) #5; gift of the Hartl lab, Harvard University) and *D. sechellia* Robertson strain (UC San Diego *Drosophila* Species Stock Center (DSSC) #14021-0248.25; gift of the Hartl lab). For hybrid complementation experiments, the following *D. melanogaster InR* loss of function lines were used: the InR^{339} hypomorphic allele (Brogiolo et al., 2001; Fernandez et al., 1995) a gift of the Hafen lab (ETH Zurich)); the InR^{GC25} inversion allele (BDSC #9554; (Chen et al., 1996)); and the *Df(3R)Exel6186* deficiency allele (BDSC #7647).

Flies were maintained on standard lab diet (32g Torula yeast, 60.5g corn meal, 128g dextrose, 9.2g agar per liter). All rearing and experiments were performed at 25°C at 60-70% humidity.

Student's *t* test was used for all pairwise comparisons of differences in means unless otherwise noted.

RNA extraction/cDNA synthesis/qPCR

Wandering third instar larvae were first sorted by presence or absence of GFP expression (GFP^+ = control hybrids containing *TM3* $P\{w^{+mC}=Act:GFP\}JMR2\ Ser^1$ balancer chromosome; GFP^- = experimental hybrids containing *InR* mutation) . Total RNA was extracted from well-fed female larvae that were grown on rich diet. RNA was extracted using Trizol (Invitrogen), treated with TURBO DNase-I (Ambion, Life Technologies), and phenol-chloroform extracted. cDNA was prepared using oligo-dT

primers and 0.5-1μg RNA per reaction with Superscript III First Strand Synthesis Kit (Invitrogen).

qPCR

qPCR was performed using PerfeCta SYBR Green SuperMix, Low Rox (Quanta Biosciences). *rp49* was used an expression control. I note here that no expression normalization control was used. For these pilot experiments, single biological replicates of 5-8 whole larvae were used for each genotype. These biological replicates were collected from two independent hybrid crosses. Each reaction was run in triplicate (technical replicates). In order to detect *InR*, the first primer was designed to be a perfect match in both species (common *InR* reverse primer, 3'-5': TCACCCCCGCTAGGTAATCAT). The complementary primer was designed to overlap sequence containing two SNPs between the reference *D. melanogaster* and *D. sechellia* sequences (*D. melanogaster*-specific *InR* forward primer: TCTGCGTGAAAGGAATTGATAATAA; *D. sechellia*-specific *InR* forward primer: TCTGCGTGGAAGGAATAGATAATAA). The primer pairs span a large (~7kb) intron. I verified species-specificity of primers via 1% agarose gel electrophoresis. The *rp49* primer pair was designed to match perfectly to the same sequence in both species (*rp49* primers, 3'-5': *rp49*-f, TGCTAAGCTGTCGCACAAATG, *rp49*-r, TTCTTGAATCCGGTGGGCAG). The cDNA template used as the standard was an equal concentration mix of cDNA from all genotypes.

Results and Discussion

Optimizing cDNA amount for qPCR reactions

The qPCR experiment was performed three times, twice using 100ng cDNA per reaction and once with 400 ng cDNA per reaction. I began with 100ng cDNA in order to maximize the potential number of experiments I could perform from a single cDNA prep. In this run I obtained poor PCR amplification efficiency. When I increased cDNA amount to 400ng per reaction, however, amplification efficiency was greatly improved, suggesting that a relatively large amount of cDNA (400ng per reaction) was necessary to obtain reliable results in this experiment. As a result, I present results for the run containing 400ng cDNA per reaction. I did find when the 100ng per reaction condition was repeated, comparable results were obtained as in the first experiment, suggesting that results are at least repeatable at this concentration. Furthermore, the broad trends that can be made from the 400ng per reaction condition are largely maintained in the 100ng per reaction condition (data not shown).

Broad technical considerations of qPCR results

The data from the run containing 400ng cDNA per reaction are presented in Table C.1. First, I note that several lanes failed to yield an observable/recordable C_T value. This result is perhaps not altogether surprising given the relatively high C_T values (C_T=31-35 for *InR*, compared to C_T=19-21 for *rp49*) obtained through this experiment, particularly for the *D. melanogaster* allele. These results could indicate several things. First, high C_T values suggest that the *InR* transcript is in much lower abundance compared to *rp49*. This result is not altogether unexpected given the different biological functions of these

proteins and previous reports looking at the expression of these transcripts in different contexts (McManus et al., 2010). Second, these data may indicate that the specific primer set used in this experiment was not particularly efficient. Although the standard curves (data not schown) indicate that this primer set is a reliable detector of relative transcript expression, these curves do not necessarily determine absolute expression level. I would suggest trying several additional primer sets to resolve this issue, which should be addressed before proceeding with this experiment.

Table C.1: Summary of allele-specific expression of *InR* in *D. melanogaster*/*D. sechellia* hybrids.

Gene	*rp49*		*InR-Dmel*		*InR-Dsec*	
	C_T, ave	95%CI	C_T, ave	95%CI	C_T, ave	95%CI
OR	19.59	0.06	34.31	1.28	32.42	0.25
Df(3R)6186 JMR2	19.75	0.03	32.52§	0.30	31.87	0.26
Df(3R)6186	19.64	0.07	33.39	0.66	30.78**	0.09
InR^{GC25} JMR2	21.14	0.04	34.05	-	34.26	0.94
InR^{GC25}	18.86***	0.05	30.97	0.36	30.39*	0.28
InR^{339} JMR2	20.06	0.15	33.49	1.02	34.04	0.80
InR^{339}	19.24**	0.06	33.28§	0.74	31.73*	0.45

Table C.1: Summary of allele-specific expression of *InR* in *D. melanogaster*/*D. sechellia* hybrids. In this experiment, 400ng of cDNA from the indicated genotype was added to each reaction. C_T values shown are averages of technical replicates of the indicated reaction within a single experiment. In one case (*InR^{GC25}-JMR2*), only a single well yielded a C_T value, and thus a confidence interval could not be determined. '*JMR2*' refers to *TM3 P{w^{+mC}=Act:GFP}JMR2 Ser^1* balancer chromosome; these are the control sisters. 95%CI = 95% confidence interval. '*' indicates statistically significant difference when comparing JMR2 control to experimental hybrid for the same gene. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. '§' indicates statistically significant difference when comparing *InR* expression level between *D. melanogaster* and *D. sechellia* within the same genotype. § $p < 0.05$.

Initial Biological Interpretations

Given the caveats addressed above, I would hesitate to make specific conclusions from these data. Nevertheless, I describe two broad conclusions that may be made from this experiment. First, there is no evidence that *D. sechellia InR* is expressed at lower levels than *D. melanogaster InR* in wild type hybrids, including both *Oregon R* and control hybrids (Table C.1). In one instance (*Df(3R)6186* controls), *D. sechellia InR* expression may actually be increased relative to *D. melanogaster* (Table C.1; C_T=32.52 for *D. melanogaster* versus C_T=31.87 for *D. sechellia*; $p<0.05$). This result is consistent with my data that suggest that in pure species (whole female larvae), *InR* expression is higher in *D. sechellia* compared to *D. melanogaster* (Chapter 4). Given the experiment as performed, however, I cannot tell the relative level of species-specific expression in hybrids compared to expression level in pure species. This may be a point to consider in future iterations of this experiment.

A second observation is that specifically for the *D. sechellia* transcript, expression levels are significantly greater in hybrids containing *InR* loss of function mutants compared to their respective controls (Table C.1). This result is not explained by differences in overall transcription, as *rp49* does not show the same trend among all genotypes. This may suggest that *D. sechellia InR* transcript is upregulated in hybrids containing a loss of function allele from *D. melanogaster*. This is an interesting result, as we previously found that hybrids harboring a loss of function *D. melanogaster InR* allele have significantly fewer TFs than do wild type hybrids (Green and Extavour, 2014). Taken together, these results would suggest that the INR protein, and not *InR* transcript expression levels, from *D. sechellia* negatively impacts TF number. This result is

consistent with other data (described in Chapter 4) that suggest that protein-coding changes, and not cis-regulatory changes, control species-specific *InR* function in *D. melanogaster* and *D. sechellia*.

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