Differential Gene Expression in the Fungus Fly, *Sciara coprophila*: The Quest for Stable Reference Genes

By
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Abstract of Differential Gene Expression in the Fungus Fly, *Sciara coprophila*: The Quest for Stable Reference Genes by Audrey Katherine Lee, ScM, Brown University, May 2017.

Strict regulatory mechanisms ensure that the cell's genome is perfectly duplicated. In eukaryotic cells, origins of replication fire once and only once per cell cycle. In the fungus gnat *Sciara coprophila*, polytene chromosomes of the salivary glands undergo locus-specific DNA re-replication, leading to DNA amplification which are visible along the polytene chromosomes as DNA puffs.

The *Sciara* genome and transcriptome resources are new, allowing exploration of *Sciara* differential gene expression for the first time. Validation of RNA transcripts that correspond to developmental events during salivary gland stages and life cycle stages may point towards biological factors critical to DNA amplification. More specifically, qPCR validation of transcripts that are abundant at the start of re-replication and decrease thereafter during salivary gland development may encode potential amplification factors that play a significant role in DNA amplification. Conversely, RNA transcripts that increase during the eye-spot stages through the end of larval development may encode proteins encoded by the DNA puffs.

Sex-specific differential gene expression was also investigated. The X' inversion is a large paracentric inversion on the X chromosome; the X' inversion is only found in females. *Sciara* female adult containing the X' inversion (XX') produce only daughters, while XX female adults produce only sons. There is no Y chromosome in *Sciara*. Inspection of the transcriptome maps might indicate which genes resides near the X' inversion breakpoints or span the breakpoints. Additionally, differences in transcript levels between male and female *Sciara* at different stages may point towards genes involved in sex determination pathways. The *Sciara* transcriptome data will be compared to *Drosophila* where the pathway for sex determination involves differential splicing of mRNA, which may be encoded by genes on autosomes, and not just on the sex chromosomes.

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Introduction

1.1 The Eukaryotic Cell Cycle

The cell cycle plays a critical role in monitoring organismal development. In eukaryotes, a cell replicates its genome and divides it into two identical copies. The cell cycle consists of the G1 phase, S phase, G2 phase and M phase (Figure 1). During S phase, the DNA is replicated, resulting in two identical sister chromatids. The sister chromatids then segregate during mitosis (M phase). The two gap phases occur between mitosis and S phase (G1), and between S phase and M phase (G2) (Novak et al., 2003). The eukaryotic cell cycle is a highly controlled process, with the major control points occurring during the transition from G1 to S phase, and the initiation of DNA replication at replication origins (Gopalakrishnan et. al, 2001). Because the cell cycle operates under strict regulatory mechanisms, successful, normal cellular division is normally ensured (Nguyen et. al, 2001). Eukaryotic cells utilize several regulatory mechanisms to ensure that replication origins are activated only once per cell cycle. The result of these tightly controlled regulations is perfect duplication of the genome (Aladjem et. al, 2007). Once DNA replication is initiated, eukaryotic cells are committed to completing the cell cycle.

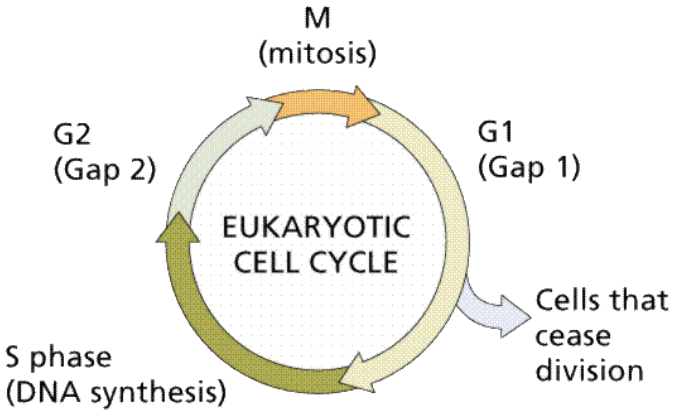


Figure 1. The eukaryotic cell cycle.

1.2 Initiation of DNA Replication

The S phase of the eukaryotic cell cycle is characterized by initiation of DNA replication. Following mitosis, the pre-replicative complexes (pre-RCs) are assembled, rendering origins replication competent. DNA synthesis begins at

specific sites, known as origins of DNA replication (ORI) (Bielinsky et al., 2001). Initiator proteins are essential to origin activity, and thus replication initiation (Jacob et al., 1963). In eukaryotic cells, the origin recognition complex (ORC) binds origins throughout the cell cycle. ORC is an initiator that loads the proteins of the pre-replication complex (pre-RC), namely, Cdc6, Cdt1 and any Mcms. The minichromosome maintenance (Mcm) proteins serve as the helicase to unwind the DNA. The initiation of DNA replication begins at the origin and proceeds bidirectionally through a highly regulated process, which includes an additional licensing step (loading the Mcm helicase) that establishes replication competence (Bielinsky et al., 2001).

DNA polymerase is recruited to each ORI once during the S phase of the eukaryotic cell cycle. The formation of the pre-RC only occurs in G1 when cyclin-dependent kinase (Cdk) levels are low. Cdks consist of a catalytic kinase subunit and a regulatory cyclin subunit, and are critical for protein-kinase activity that molecularly regulates cellular division (Heller et al., 2011). Cdk activity increases at the end of G1, allowing passage of the pre-RC through the G1 commitment point. Progression through this checkpoint triggers Cdc6 and Cdt1 to leave the pre-RC, and activates Cdc7-Dbf4 and Clb-Cdc28. The activation of the latter kinases trigger origin unwinding, replication fork machinery assembly, initiation of daughter strand synthesis, and conversion of the pre-RC into the initiation complex (IC) (Nguyen et al., 2001). Formation of the IC requires Cdc45 and GINS to bind to the Mcm2-7 complex. In turn, helicase activity is activated in the Mcm2-7 complex, thus allowing origin firing to occur and initiate DNA synthesis (Ilves et al., 2010).

1.3 DNA Re-replication and DNA Amplification

During the remainder of interphase, Cdk activity remains high, which inhibits IC formation from a new pre-RC. However, an override of cellular regulatory control mechanisms leads to multiple firings of the origin of replication. Deregulation of cellular checkpoints results in DNA re-replication, which then leads to DNA amplification. The onion-skin model for DNA re-replication was first

proposed by Botchan and co-workers in 1979 (Figure 2). The initiation of new replication forks before the leading fork completes DNA synthesis results in replication forks within replication forks (Botchan et al., 1979). This model can be clearly visualized by Miller spreads through electron microscopy in the flies *Drosophila* (Osheim et al., 1988) and *Sciara* (Figure 2, unpublished data from the Gerbi laboratory).

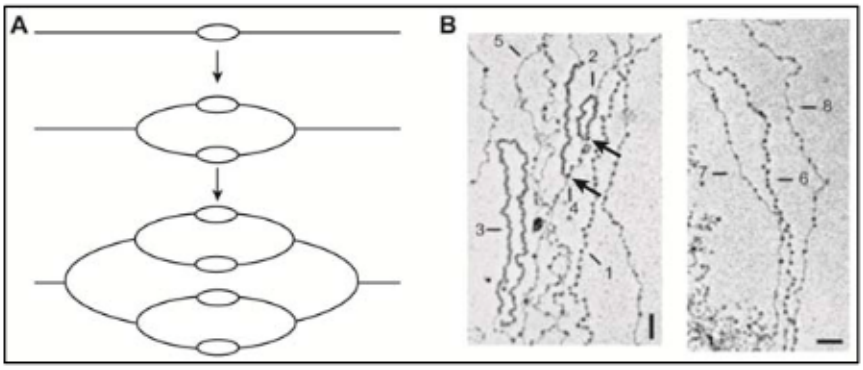


Figure 2. (A) Onion skin model of re-replication. (B) Electron micrographs of Miller spreads with big arrows depicting nested replication forks, consistent with the onion-skin model of DNA re-replication.

It is evident that re-replication is a potential basis for DNA amplification, but the mechanism for initiation of site-specific DNA amplification remains unclear. This phenomenon of DNA amplification is a hallmark of cancer, where certain loci re-replicate, resulting in extra copies of those loci in the genome and ultimately, genomic instability (Blow et al., 2008). While it is difficult to elucidate the underlying mechanism of DNA re-replication and DNA amplification in cancer cells, there are two known cases in which the re-replication controls are overridden at specific loci and occurs naturally during development (Nordman et al., 2012). Under normal developmental conditions, intra-chromosomal DNA amplification occurs in late larval *Sciara coprophila* salivary gland polytene chromosomes, and chorion genes in *Drosophila melanogaster* follicle cells. It is presumed that DNA amplification occurs in these two systems to produce a large amount of protein in a short period of time (Kim et al., 2011). In *Sciara*, large amounts of protein are needed for the pupal case (Wu et. al, 1993). In *Drosophila*, DNA amplification is employed to produce protein for the eggshell

(Xie et al., 2008). Mitosis does not occur in the polytene cells undergoing DNA amplification in these organisms, which allows the onion-skin structure to persist. Additionally, in both systems, DNA amplification occurs in tissues that are eliminated in the next developmental stage. As a result, these organisms do not live with the consequences of DNA amplification. This process requires normal replication machinery, including necessary components of the pre-RC. During amplification, cyclin E is present at high levels. These increased levels of cyclin E repress thousands of ORIs throughout the genome, with the exception of origins involved in DNA amplification (Calvi et al., 1998).

1.4 Significance of DNA Amplification

DNA replication is associated with several human diseases, including multiple different syndromes, and cancers (DePamphilis et al., 2006). Some of these diseases correlate with defects in the initiation of replication, while other diseases are linked to defects in the elongation of DNA synthesis. In patients with Meier-Gorlin syndrome, which leads to dwarfism, mutations in proteins critical for DNA initiation have been identified. Specifically, loss of function mutations in pre-replication complex proteins, including ORC, Cdc6, and Cdt1, have been found in these patients prone to dwarfism (Bicknell et al., 2011). The final step in formation of pre-replication complexes is the recruitment of the MCM proteins, which license each ORI. It has been shown that the large majority (80% to 100%) of malignant and high-grade premalignant lesions are Mcm immunopositive. As a result, it has been suggested that cancer cells are continuously licensed for DNA replication (Jackson et al., 2014). High levels of Mcm can lead to DNA re-replication, which may be an initial step in DNA amplification. DNA re-replication results in nested replication forks that are susceptible to breakage, leading to chromosome instability. While the occurrence of DNA amplification is well-known in many cancers, the mechanism for its initiation is poorly understood.

1.5 Development and Maintenance of Sciara coprophila

The lower dipteran fungus gnat *Sciara coprophila* develops through four instar larval stages followed by pupation to become an adult (Buck, 1937). The cuticle is shed at the end of each instar. The life cycle is a duration of five weeks. Between seven to ten days after egg-laying, the embryos emerge as larvae. The first three larval instars occur immediately following post-hatch up until two weeks after post-hatch. These instars are characterized by small body size, and lack of eyespots. The fourth larval instar stage begins approximately two weeks post-hatch, and proceeds for about seven to ten days. Mid-way through the fourth instar stage, “eyespots” develop, which are located at the anterior end of the larvae, and eventually become the adult eyes. *Sciara* larvae are categorized into six eyespot stages. These stages are critical to this research, as they visibly mark the developmental progression of DNA amplification in *Sciara* salivary glands (Wu et al., 1993). The eyespot stages are characterized by the number of rows and columns of eyespot granules making up the larval eyespots (Gabrusewycz-Garcia, 1964). The early eyespot stage of 8x4 is followed by 10x5, 12x6, 14x7, edge-eye, and finally, drop-jaw (Figure 3). DNA puff amplification in salivary gland polytene chromosomes occurs during the later eyespot stages. Following the fourth instar larval stage, pupation takes place for a few days. During pupation meiosis occurs. Subsequently, adult flies emerge, and live for approximately one week. Males and females mate, followed by embryo laying, thus continuing the *Sciara* lifecycle.

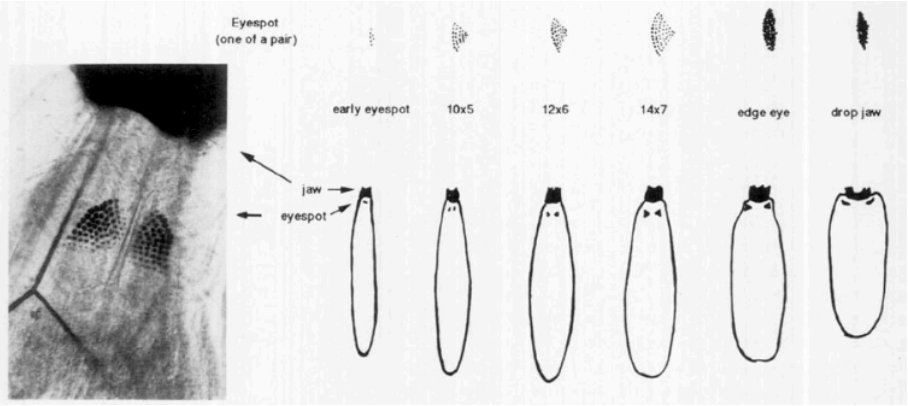


Figure 3. Eyespot stages in *Sciara* fourth instar larvae. Morphological differences in larval body shape, and eyespots are clearly illustrated over developmental time (Wu et al., 1993).

1.6 Sciara coprophila *as a Model System*

While *Drosophila melanogaster* was chosen as the leading model organism in the 1930s at a Cold Spring Harbor meeting, *Sciara coprophila* serves as a unique biological system. This lower Dipteran fly exhibits biological properties that are distinct to *Sciara*. The biological features of *Sciara* most relevant to this thesis research can be divided into two groups: chromosome dynamics and DNA replication. In the realm of chromosome dynamics, *Sciara* house a remarkable ability to differentiate between maternally inherited and paternally inherited chromosomes. This phenomenon of marking chromosome ancestry is termed chromosome “imprinting” and was first coined in *Sciara* (Crouse, 1960b). Chromosome imprinting is essential to sex-specific chromosome elimination events during embryogenesis and spermatogenesis. The chromosome elimination events observed in *Sciara* are regulated by the “controlling element” (CE), which is a region at the end of the X chromosome and residing within approximately 50 rDNA copies. Through translocation studies, the CE has been mapped to a region of heterochromatin proximal to the X chromosome centromere and within the tandem array of rDNA repeats (Crouse et al., 1977). Finally, *Sciara* contain a set of germline limited (L) chromosomes that are specific to the germline. Not much is known about the function of L chromosomes, but they likely serve to distinguish the germline from the soma during early development. These biological phenomena are just a few

characteristics observed in this system that will aid in our understanding of basic genetic, cellular, and developmental biological mechanisms.

Additionally, there are many experimentally favorable aspects of *Sciara* that make it a valuable model system. Firstly, larvae are able to be stored for numerous months at 4°C, without maintenance. During these long periods of storage, larval growth is arrested. Mothers also yield only sons or only daughters, negating the need to collect virgins for mating. Time-laid embryos are easy to collect and serve as a valuable tool for studying early embryogenesis in *Sciara*. Furthermore, *Sciara* salivary glands extend the full length of the larval body, providing plenty of experimental material for DNA amplification studies. The salivary glands also give rise to gigantic polytene chromosomes, providing ample substrate for cytology and prominent visualization for microscopy. Finally, *Sciara* polytene chromosomes lack a chromocenter, which is a dense mass of centromeric heterochromatin holding together the *Drosophila* chromosomes. The absence of a chromocenter allows easier spreading of *Sciara* chromosomes, making it easier to identify each unique salivary gland chromosome (3 autosomes and one X chromosome).

As a model organism, *Sciara coprophila* provides researchers with a unique system to study gene amplification, sex determination, the X chromosome controlling element (CE), chromosome imprinting and many other facets of *Sciara* biology.

1.7 DNA Puff Amplification

Like *Drosophila*, *Sciara* possesses both polytene and diploid tissues. Polytene chromosomes are oversized chromosomes present in the salivary glands of *Sciara*. Polytene chromosomes result from endoduplication (many rounds of replication without intervening mitosis). While daughter chromatids do not synapse in polyploid cells, the daughter chromatids remain synapsed together in polytene chromosomes. As a result of these synapsed chromatids, giant chromosomes are observed. The *Sciara* model system undergoes developmentally regulated DNA re-replication, leading to DNA amplification at

‘DNA puff’ loci (Crouse et al., 1968). The gigantic *Sciara* polytene chromosomes undergo twelve rounds of endoduplication in the larval salivary glands (Rasch, 1970). This phenomenon results in 4,906 aligned sister chromatids (or 4,096c, where c is the haploid DNA content). Intense DNA transcription occurs at these puff locations along the salivary gland polytene chromosomes as a result of the amplified DNA that provides increased template for transcription (Wu et al., 1993). The DNA puffs can be viewed through microscopy as swellings at specific loci.

The largest DNA puff present on *Sciara* salivary gland chromosomes, puff II/9A (Figure 4), amplifies approximately 16-fold (Foulk et al., 2006). Puff II/9A has been extensively studied. The II/9A locus contains two transcription units that undergo RNA synthesis activated by a hormone, ecdysone (Bienz-Tadmor et al., 1991). Miller spreads of *Sciara* salivary gland chromosomes that have undergone amplification support the onion-skin model for DNA amplification, illustrated by visible nested replication forks (unpublished data from the Gerbi laboratory).

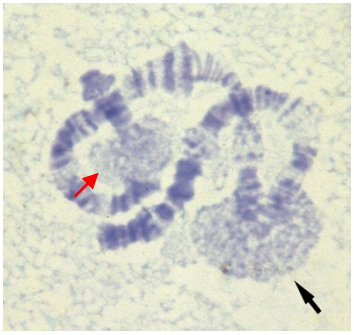


Figure 4. DNA puff II/9A (black) and DNA puff II/2B (red) of *Sciara* salivary glands.

Validation of RNA transcripts that correspond to developmental events during salivary gland stages and life cycle stages may point towards biological factors critical to DNA amplification. More specifically, qPCR validation of transcripts that are abundant at the start of re-replication and decrease thereafter during salivary gland development may encode potential amplification factors that play a significant role in DNA amplification. Conversely, RNA transcripts that

increase during the eye-spot stages through the end of larval development may encode proteins encoded by the DNA puffs.

1.8 The Sciara *Genome*

The diploid *Sciara* genome is composed of 3 pairs of autosomes, 2 sex chromosomes and 2 germline limited chromosomes. The autosomes are named chromosomes II, III and IV, and undergo locus-specific DNA re-replication and amplification in the salivary glands (Figure 5). *Sciara* lack a Y sex chromosome, but contain two types of X chromosomes: X and X'. The X' chromosome contains a large paracentric inversion that excludes the centromere (Crouse, 1977). This particular chromosome is present in some *Sciara* females. Female adults that contain this inversion are XX' and produce only daughters, while XX female adults produce only sons (Metz and Schmuck, 1929).

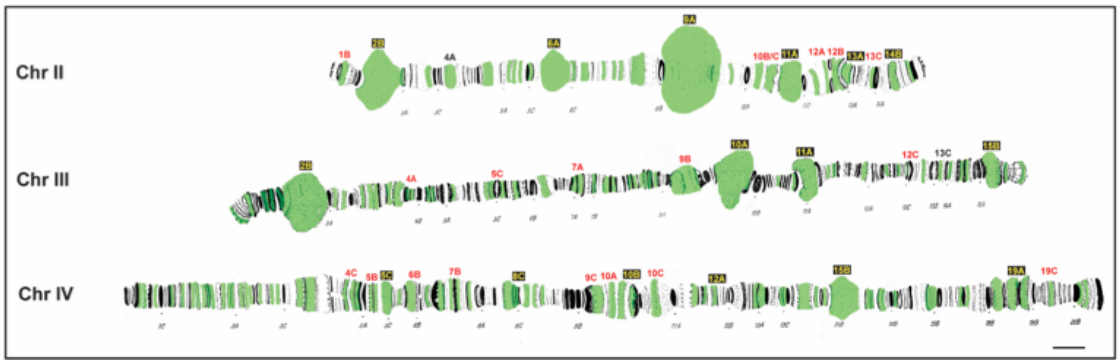


Figure 5. Chromosome maps illustrating autosomes from the *Sciara* salivary glands (Gabrusewycz-Garcia, 1964, redrawn by Liew et al., 2013).

1.9 Chromosome Dynamics and Current Understanding of Sex Determination in Sciara

Unlike *Drosophila*, which exhibits sex-specific chromosome ratio differences as a zygote, *Sciara* male and female embryos both start with the same 3X:2A chromosome ratios. *Sciara* undergo distinct, tissue-specific dynamic chromosome events that are critical to sex determination and development: paternal X chromosome (Xp) elimination in the soma, Xp elimination in the germline, paternal chromosome elimination during meiosis I of spermatogenesis, X nondisjunction during meiosis II of spermatogenesis and germline limited (L)

chromosome elimination from the soma (Figure 6). L chromosome expulsion occurs identically in the soma of both sexes and will not be the focus of sex-specific chromosome elimination events.

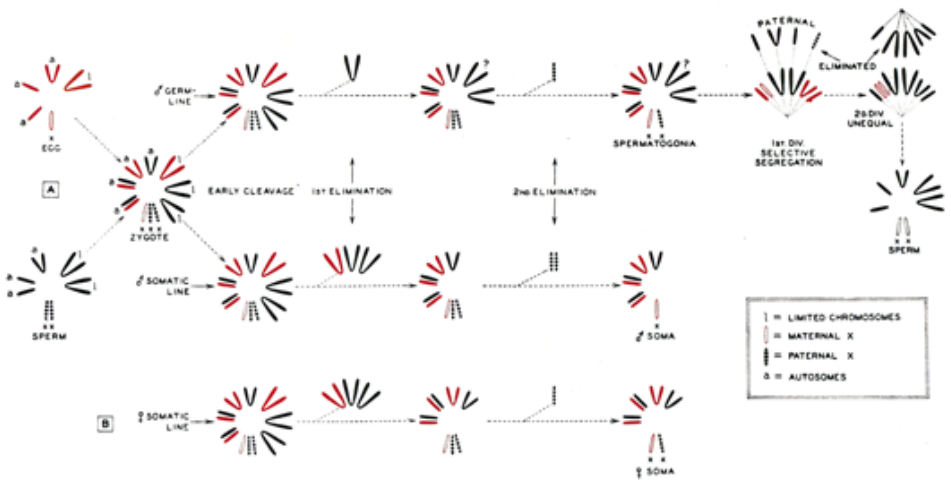


Figure 6. Unique, developmentally regulated chromosome elimination events during early embryogenesis and spermatogenesis in *Sciara* (Gerbi, 1986).

Between the 7th and 8th cleavage of embryogenesis, somatic cells undergo an Xp elimination event in which one Xp chromosome is eliminated in females and two Xp chromosomes are eliminated males. While the genome and transcriptome data have recently become available, some aspects of sex determination and chromosome elimination in *Sciara* have been well studied through immunofluorescence microscopy. Through fluorescence microscopy, investigators revealed the cytological events that characterize this particular chromosome elimination event. During anaphase in *Sciara* somatic cells, chromosomes begin their movement towards the poles. The future eliminated Xp chromosomes are incapable of complete separation and remain at the equatorial plate.

In germ cells, one of the two Xp chromosomes is expelled from all germ cells in both sexes. This elimination occurs during the “resting stage” in which germ cells are located in the gonad site, but remain undivided until the second instar larval stage. The Xp chromosome is eliminated at any time during this period of stasis. During elimination, it is hypothesized that one Xp remains

closely attached to the nuclear membrane. That particular Xp is then excised from the cytoplasm through a nuclear “bulge” (Goday and Esteban, 2001). The exact mechanism of this elimination event, including how one of the two Xp chromosomes are targeted for expulsion are unknown.

Meiosis in *Sciara* occurs during the pupal stage of the lifecycle. While females undergo normal meiosis, males experience aberrant male meiosis. During meiosis I of spermatogenesis, all paternal chromosomes are eliminated through a cytoplasmic bud. A monopolar spindle directs segregation of the maternal chromosomes to a single pole. The paternal chromosomes move in the opposite direction away from the single pole and enter a cytoplasmic bud, which is eventually excised from the future spermatocyte. The remaining chromosomes of the future sperm nucleus are maternally inherited. During meiosis II, the two chromatids of the maternal autosome (Am) segregate normally. In contrast, the two chromatids of the maternal X (Xm) chromosome undergo X nondisjunction in which the chromatids do not segregate. The maternal autosomes align at the metaphase plate, but the undivided Xm chromosome remains at one pole of the spindle. In telophase of male meiosis II, the nullo-X group of chromosomes is eliminated in a bud of cytoplasm. Thus, male meiosis in *Sciara* produces only one spermatocyte that will form the future sperm. The resulting sperm is exclusively composed of maternal genetic material. At some point during development, the maternal chromosomes of the spermatocyte are marked as paternal. Chromosome imprinting marks the fate of that chromosome, which will either be eliminated or retained. The mechanism through which this curious imprinting event occurs is unknown (Sanchez, 2008).

While the molecular events responsible for chromosome imprinting are unclear, there is data to support histone modifications as a potential *Sciara* imprinting mark. Goday et al. (2001) clearly demonstrated that histone modifications and chromatin organization are correlated with chromosome elimination to some extent. In *Sciara* somatic cells, both maternally derived chromosomes and paternally derived chromosomes are hyperacetylated. As a result, there is no significant difference in acetylation patterns that distinguishes

the eliminated Xp chromosome. In contrast, chromosomes of *Sciara* germline cells exhibit differences in acetylation and methylation patterns depending on their inheritance (Goday et al., 2002). Maternally derived chromosomes in the germline are hypoacetylated and hypermethylated, while paternally derived chromosomes are hyperacetylated and hypomethylated, exhibiting opposing modifications. Interestingly, the Xp eliminated from the germline cells in both sexes is hypoacetylated and hypermethylated (Table 1). The excised Xp chromosome shares similar acetylation and methylation patterns to the maternally derived chromosomes. These findings suggest that there is an additional layer of cellular and molecular regulation that distinguishes the Xp chromosome for elimination. An explanation for this precise elimination mechanism may lie in sex-specific differential gene expression.

Histone modifications during *embryonic* chromosome elimination				
	Somatic Cells		**Germ Cells**	
Chromosome	Acetylation		Acetylation	Methylation
A_m	+		-	+
A_p	+		+	-
$X_{p(eliminated)}$	+		-	+

Table 1. Histone modifications affecting *Sciara* paternal chromosome elimination embryogenesis.

Similarly, male germline cells undergo a series of histone modifications during meiosis I of spermatogenesis. Maternally derived chromosomes are hyperacetylated and hyperphosphorylated at prophase I. By anaphase I, these chromosomes are hypophosphorylated. In contrast, paternally derived chromosomes are hypoacetylated and hyperphosphorylated at anaphase I. The hyperphosphorylation persists until a complete set of paternal chromosomes is eliminated. By meiosis II, after the paternally derived chromosomes are eliminated from the future sperm nuclei, the maternally derived chromosomes are dephosphorylated (Escriba et al., 2013). While the role of histone modifications in *Sciara* chromosome elimination are unclear, differential gene expression of

kinases, phosphatases, deacetylase, acetyltransferases and/or methyltransferases between male and female embryos might further elucidate the mechanisms controlling observed similarities and differences seen in chromosome modifications.

Histone modifications affecting chromosome elimination during *spermatogenesis*				
	1st meiotic division			**2nd meiotic division**
		At prophase:	During anaphase:	
Chromosome	Acetylation	Phosphorylation	Phosphorylation	Phosphorylation
A_m	+	+	-	+, then -
A_p	-	+	+	(X_p eliminated)

Table 2. Histone modification patterns affecting paternal chromosome elimination during spermatogenesis.

The X chromosome controlling element (CE) regulates X chromosome elimination during early embryogenesis and X nondisjunction during male meiosis. The CE controls centromere activity and has been mapped to a region of the X chromosome containing heterochromatic blocks proximal to the centromere. The role of the CE was determined through a series of carefully conducted translocation experiments in which regions of the X chromosome and regions of autosomes were swapped in reciprocal translocations. Following the translocation of the CE and autosomal regionals, X chromosomes without the CE behaved like autosomes and failed to undergo elimination. In contrast, autosomes containing the CE behaved like typical X chromosomes and underwent elimination. These results provide clear evidence that there is a particular region of the X chromosome that regulates chromosome elimination in *Sciara* (Crouse, 1960). Despite the body of evidence that supports the existence and function of the CE, many questions regarding its mechanisms of action yet to be answered: How is the CE able to act across long distances? Is the CE a single gene, group of genes or long non-coding RNA?

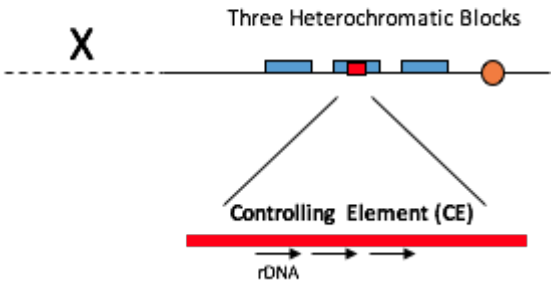


Figure 7. Schematic of the X chromosome controlling element, which contains ~50 copies of rDNA within the terminal heterochromatin proximal to the centromere (adapted from Susan Gerbi).

1.10 Potential Models for Sex Determination in Sciara

The molecular mechanisms of sex determination in *Sciara* are unknown. Elucidating the complex cellular and molecular events involved in *Sciara* sex determination requires an understanding of sex determination in other insect model organisms.

The fruit fly:

Sex determination mechanisms in the fruit fly *Drosophila melanogaster* have been thoroughly studied and well-characterized. In *Drosophila*, sex determination begins with chromosome differences between male and female zygotes. Females are 2X:2A, while males are X:2A, where X represents X chromosomes and A represents autosomes (Sanchez, 2008). Following the sex-specific distinction in chromosomes, a sex determination genetic cascade begins. The cascade is controlled by the Sex-lethal (Sxl) protein, which is only functionally present in females. As a result of sex-specific alternative splicing, males possess a non-functional form of Sxl that contains a stop codon in exon 3. Despite sex-specific differences in Sxl functionality, *transformer* (*tra*) RNA is transcribed in both males and females. The *tra* pre-mRNA contains two alternative 3' splice sites. In females, functional Sxl intervention prevents a stop codon from being included in the mature transcript, resulting in functional Tra protein. In males, non-functional Sxl protein is unable to remove the stop codon in Tra protein, resulting in non-functional Tra protein. *Sciara* species contain transformer-2 genes, which were found to be transcribed in both the soma and

germline of males and females during development and adult life. *Sciara transformer-2* produces a single primary transcript and follows the same alternative splicing in both sexes, giving rise to different *tra-2* mRNA isoforms. Interestingly, the expression of *Sciara* Tra-2 protein in *Drosophila* XX pseudomales lacking the endogenous *transformer* function caused partial feminization. Although *Sciara* transformer RNA isoforms are the same in both sexes, the *Sciara-Drosophila* Tra-2 protein shows a conserved role in Drosophila sex-determination (Martín et al., 2011)

Following Tra protein in the *Drosophila* sex determination genetic cascade is *doublesex* (*dsx*). Similar to *tra*, *dsx* is transcribed in both sexes. Females express Dsx(F), while males express Dsx(M). Both Dsx proteins are transcription factors that share an N-terminal domain, but differ in the C-terminal domain, which results from sex-specific splicing of the primary *dsx* transcript. Male-specific splicing of *dsx* is the default mode since males do not have functional Tra present to alter *dsx* splicing. The incorporation of a female-specific exon caused by activation of a splice acceptor site by Tra-Tra2 complex binding results in Dsx(F).

Fruitless (*fru*) functions at the bottom of the sex determination genetic cascade. Similar to the role of Tra complexes in *dsx* sex-specific splicing, the functionality of Tra-Tra2 complexes dictate alternative splicing in *fru*. In females, the functional Tra-Tra2 complex binds target sites along the *fru* mRNA transcript. A female-specific exon containing a stop codon is included in the mature mRNA, preventing functional Fru protein from being translated. Since functional Tra protein is absent in males, the female-specific exon is spliced out resulting in functional FruM protein. This protein is essential in male-specific *Drosophila* development, including CNS development and male courtship behavior[1] (Gempe and Beye, 2011).

Although Sxl controls sex determination in *Drosophila*, Sxl homologs in other insect species do not serve as potent regulators of sex determination. *Sciara* contain a single isoform of Sxl present in both sexes, suggesting the protein's sex-independent function. *Drosophila* sex determination is established

through a pattern of sex-specific alternative splicing that affects downstream sex determination genes. Comparison to genes involved in *Drosophila* sex determination can identify the *Sciara* homologs and any differential gene expression levels between females and males throughout *Sciara* development can be explored. Additionally, patterns of alternative splicing in *Sciara* can also be explored as potential modes of differential gene expression. Using *Drosophila* as a model, *Sciara* homologs of other sex determination genes and their alternative splicing may serve as important clues to sex determination in *Sciara*.

The honeybee:

In some hymenopteran insects, such as wasps, ants and bees, sex is regulated by haploidy/diploidy. Through this mechanism, males are haploid and develop from unfertilized eggs, while females develop from fertilized eggs. In the honeybee *Apis mellifera*, the underlying genetic mechanism responsible for haploid/diploidy sex determination was further elucidated. The complementary sex determination (csd) gene was isolated from this honeybee and shown to be transcribed in both sexes. However, the csd gene has a highly variable region responsible for allelic variation and various Csd isoforms. It has been hypothesized that this allelic heterozygosity generates two functional Csd protein isoforms capable of forming a complex that determines female development. In contrast, allelic homozygosity produces a single Csd protein isoform incapable of forming a functional complex, resulting in male development. In the honeybee system, males from unfertilized eggs are hemizygous at the csd locus while females are heterozygous at the same locus (Beye et al., 2003). Using this organism as a model system, a similar heterozygous complex formed in X'X *Sciara* females might be involved in determining female development. Perhaps XX' females generate two forms of a sex determination protein that leads to female development, while XX females fail to produce such a protein, resulting in male development. Validating differences in gene expression levels between female-producing mothers and male-producing mothers may point towards

genes that are responsible for *Sciara* sex determination through a similar locus-specific manner.

The scale insects:

In coccids, paternal chromosome inactivation or elimination occurs to distinguish males from females. Within this family of insects, there is a distinction between the lecanoid mechanism and the diaspidoid mechanism. In lecanoid coccids, sex determination is based on a functional haploidy/diploidy mechanism. Immediately following fertilization, all chromosomes of the embryo are euchromatic. Later in development, paternally derived chromosomes become heterochromatic in male-destined embryos. In contrast, female embryos retain active chromosomes from both parents, as the paternal chromosomes fail to become heterochromatic. As a result, females have two functional chromosomal complements, while males are structurally diploid but functionally haploid. In diaspidoid coccids, sex determination is also based on a haploid/diploid mechanism. However, male embryos of diaspidoid coccids undergo paternal chromosome elimination in which all paternally inherited chromosomes are removed from cells. Female diaspidoid coccids retain functionality of chromosomal complements inherited from both parents. The resulting diaspidoid coccid males are structurally and functionally haploid, while their female counterparts are structurally and functionally diploid (Herrick and Seger, 1999). Similarly, the *Sciara* soma and germline undergo sex-specific paternal chromosome elimination. Might *Sciara* and coccids share similar mechanisms of chromosome imprinting?

1.11 Master's Thesis Project

Genome and transcriptome resources have recently become available for the fungus fly, *Sciara coprophila*. This provides the opportunity to investigate gene expression for the first time in *Sciara*. The purpose of this research project is to use quantitative PCR (qPCR) of total RNA extracted from female salivary glands and sex-specific stages of *Sciara* development to confirm patterns of gene expression mined from RNA-sequencing (RNA-seq) data.

Differences in salivary gland gene expression levels across the eyespot stages may point towards transcripts and proteins involved in mediating locus-specific DNA re-replication and amplification. Guided by the mysterious nature of chromosome elimination and sex determination in *Sciara*, differences in gene expression levels across the lifecycle stages of males and females may point towards genes involved in sex determination.

In order to validate developmental and differential expression patterns pulled out of RNA-seq data, qPCR on total RNA from staged female salivary glands and sex-separated life stages (embryos, larvae, adults) will be performed to determine differences in gene expression. Experimental material was collected from dissected female staged salivary glands (≤8x4, 10x5, 12x6, 14x7, edge eye/drop jaw), and sex-specific whole embryos, larvae, pupae, and adults.

Specific primer pairs were designed for an array of reference genes. Potential reference genes were validated in order to determine a developmentally stable reference gene found at constant expression levels to be used in differential gene expression qPCR experiments. Primer validation plates and test plates were completed using qPCR and the results were analyzed using an RStudio script.

Methods

2.1 qPCR of DNA Puffs

qPCR validation experiments were performed on DNA puffs derived from sequencing data. *Sciara coprophila* were maintained in the laboratory at 21°C. Female fourth instar larvae, which undergo one additional endocycle and have larger polytene chromosomes, were staged by eyespots using compound microscopy. The developmental stage of late fourth instar larvae was determined by counting the number of pigment granules in the eyespots of each larva (Gabrusewycz-Garcia, 1964). For each eyespot stage (≤ 8x4, 10x5, 12x6, 14x7, edge eye/drop jaw), 15 pairs of salivary glands (in three rounds of 5 pairs) were dissected in 250-300 µl of Robert's Buffer (87 mM NaCl, 3.2 mM KCl, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Tris-HCl; pH 7.3) and transferred immediately into

100 µl of DNAzol (Invitrogen) on ice. Staged salivary glands were collected in triplicates. Salivary gland DNA was extracted using the Invitrogen DNAzol reagent protocol (200 µl DNAzol total, 2 µl RNase A, 2 µl Proteinase-K, 100 µl 100% molecular grade EtOH, 100-400 µl 10 mM Tris-HCl) and stored at -20°C. Similarly, female adult genomic DNA, used as a calibrator in qPCR, was extracted using the DNAzol reagent protocol, diluted to 1 ng/µl, and stored at -20°C.

The first test plate was used to determine the concentration of DNA (50 pg/µl or 100 pg/µl) was sufficient for successful qPCR. Female adult genomic DNA was diluted to 50 pg/µl and 100 pg/µl and used as the calibrator sample. Similarly, the test samples (post-amplification salivary gland DNA) were diluted to 50 pg/µl and 100 pg/µl. Following this initial test plate, the DNA concentration used for both the calibrator and test samples was 50 pg/µl.

qPCR was performed in triplicate using DNA from edge eye/drop jaw salivary glands as the test sample. Using 6 reactions per validated primer set, 8 control sites and 14 target sites were assayed. Primer set master mixes were prepared in a total volume of 13 µl (7.5 µl 2X SYBR Green, 2.5 µl ultrapure water, 1.5 µl forward primer, 1.5 µl reverse primer) and 2 µl of the appropriate DNA samples were added to the wells according to a matrix map. Initial analysis of each plate came from the amplification plots and dissociation curves produced during the qPCR run. Further analysis of each plate considered outliers indicated by the amplification plots and dissociation curves and was performed using RStudio scripts designed by John Urban, Ph.D.

2.2 Testing qPCR recipes

A 10 µl recipe was tested using a primer validation plate of six primer pairs. The original 15 µl recipe was modified in order to minimize the use of costly reagents. Primer set master mixes for three control loci and three target loci were prepared in a total volume of 10 µl (5 µl 2X SYBR Green, 1 µl ultrapure water, 1 µl forward primer from 1 µM stock, 1 µl reverse primer from 1 µM stock). The volume of DNA was kept at 2 µl at a concentration of 50 pg/µl. The same analyses described previously were performed for the 10 µl recipe, with the

exception of adjusting the RStudio scripts according to the new volumes used in the primer set master mixes.

Additionally, a 5 µl recipe was tested using a primer validation plate of six primer pairs. The original 15 µl recipe was further modified in order to minimize the use of costly reagents. Primer set master mixes for three control loci and three target loci were prepared in a total volume of 5 µl (1 µl 2X SYBR Green, 1 µl ultrapure water, 1 µl forward primer from 1 µM stock, 1 µl reverse primer from 1 µM stock). The volume of DNA was changed to 1 µl at a concentration of 50 pg/µl. The same analyses described previously were performed for the 5 µl recipe, with the exception of adjusting the RStudio scripts according to the new volumes used in the primer set master mixes.

2.3 Preliminary TRIzol RNA extraction of mixed stage female pupae and salivary glands

Before preparing total RNA samples from staged salivary glands and life cycle stages separated by sex, a trial TRIzol RNA extraction was performed on a sample of female mixed stage pupae that was stored at -80°C. The ThermoFisher Scientific TRIzol reagent protocol was followed (500 µl TRIzol, 250 µl chloroform, 250 µl isopropanol, 500 µl 75% ethanol)

Following the total RNA extraction, a Nanodrop reading was obtained using the RNA-40 setting. A blank measurement was taken using 2 µl ultrapure water, followed by a measurement taken using 2 µl of the RNA sample. Reliable Qubit reagents were not available to determine an accurate RNA concentration. In lieu of a Qubit reading, a 5 µl aliquot of RNA mixed with 2 µl of 6X staining buffer was run on a 2% agarose gel (2 g agarose, 100 mL 1X TAE buffer) using gel electrophoresis. The first lane was loaded with 10 µl of 1 kb dsDNA ladder and the second lane was loaded with a 500 ng RNA sample. The gel was run at 105V for 1.5 hours, followed by an EtBr staining and de-staining step and ending with gel visualization under various exposure times.

Earlier qPCR validations performed on *Sciara* DNA puffs concluded that 15 pairs of female salivary glands per biological replicate yielded enough DNA for

multiple qPCR test plates. *Sciara* females undergo an additional round of endoduplication relative to *Sciara* males. As a result, female salivary glands were used to investigate genes involved in DNA amplification. To start, 15 pairs of female salivary glands (≤ 8x4) were dissected in Robert's Buffer (87 mM NaCl, 3.2 mM KCl, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Tris-HCl; pH 7.3) and carefully transferred to an Eppendorf tube containing 100 ul TRIzol. Total RNA was extracted from the salivary glands by following the procedure by ThermoFisher Scientific (250 µl chloroform, 250 µl isopropanol, 500 µl 75% ethanol). In order to validate the Nanodrop reading obtained using the RNA-40 setting, a Qubit reading was attempted. Using the Qubit RNA buffer, dye and standards available, a successful Qubit calibration was not obtained. Gel electrophoresis using a 2% agarose gel, 1 kb dsDNA ladder, and 500 ng RNA sample was used to validate the RNA integrity.

2.4 Determining average mass of larvae, pupae and adults

After performing a successful practice TRIzol total RNA extraction, the average mass of female and male larvae, pupae and adults was measured. An empty Eppendorf tube was weighed and 25 mixed stage female larvae were added. The Eppendorf tube containing larvae was weighed again and the average weight of a single larva was determined by dividing the difference between the filled and empty Eppendorf tube by 25. The same procedure was followed for male larvae, female pupae, male pupae, female adults and male adults. It was especially important to separately determine the individual mass of female and male adults as female adults are physically larger than their male counterparts. By determining the mass of an individual larva, pupa, and adult for both females and males, the approximate total number of organisms from each stage needed to reach 25 mg per sample for a single biological replicate was decided.

2.5 Sex-specific embryo plating and time laid collection

Embryo collection at various stages of embryonic development proved to be a long task. For both sexes, 25 mg of tissue per biological replicate was collected over four different embryo lay times (2 hours, 1 day, 2 days, 3 days old). There was stark variation in the amount of embryos obtained from each embryo plating experiment; some embryo plates resulted in almost 19 mg of embryonic tissue, while other plates resulted in 4 mg of embryonic tissue.

Fortunately, one of the unique biological aspects of *Sciara* is that mothers produce all female or all male offspring. From Monday through Thursday each week over the course of a semester, Jacob Bliss generated mass mating vials for both male and female embryos each day, consisting of 10 female adults and 6 male adults. These mass mating vials were maintained at 21°C overnight, and embryo plating was performed the following day from Tuesday through Friday.

Before beginning embryo plating, both large and small agar plates (22 g Bacto-Agar in 1 L solution) containing 10 mL/L of a 100X HyClone anti/anti solution were prepared. Working dose aliquots of the HyClone anti/anti solution were stored at -20°C (10 mL aliquots in 15 mL conical tube).

The dissecting microscope housed under a hood and immediate workspace and tools were sterilized using a 70-80% ethanol solution. A sterile work environment was maintained throughout the embryo plating procedure. Using a CO_2 gun and pad, adults from the mass mating vials were put to sleep. If embryos were laid in the mass mating vials, they were gently collected with a sterilized paintbrush and placed at the top of a large, labeled agar plate. The male adults, identified by their smaller size and characteristic semi-circular clasper structure at the posterior end, were collected using forceps and collected in a pre-weighed Eppendorf tube. The female adults, identified by their larger side and presence of a linear ovipositor at their posterior, were gripped by their thorax using forceps and plated on a large agar plate, beneath the embryos collected from the mass mating vials, row by row. The plated female adults and embryos were incubated in a sterile environment for at least two hours and no longer than four hours. After the first hour of incubation, forceps were used to

squeeze the thoraces that might have been missed in the initial plating. The female adults were carefully collected and placed in a pre-weighed Eppendorf tube. The Eppendorf tubes containing male and female adults were stored at -80°C for future experiments. The embryos that remained on the large agar plate were carefully collected with a paintbrush and transferred to a small, labeled agar plate. These plates were incubated in an empty, autoclaved tip box for 2 hours, 1 day, 2 days, or 3 days. Once the time laid incubation was complete, the embryos were collected in a labeled Eppendorf tube and stored at -80°C.

2.6 Collecting sex-specific, mixed stage samples across lifecycle

After determining the average weight of the tissue types needed to be collected for a total of 25 mg per biological replicate, larvae, pupae, and adults, separated by sex, were collected. A mixed stage sample consists of a random, but equal amount of tissue across a particular life stage.

Larvae were collected from sex-specific mass mating jars that were reared at 21°C in the laboratory. Due to variation in developmental stages, it was unlikely that enough larvae of one particular stage was available for 3 biological replicates that were representative of mixed stage larvae. Instead of collecting 5 larvae from five pre-determined stages (pre eyespot, early eyespot, mid eyespot, late eyespot, edge eye and drop jaw) for a total of 25 larvae and approximately 25 mg of tissue based on the earlier average weight determination, 5 larvae from one of the five eyespot stages (i.e. pre ES only, early ES only, etc.) were collected in a labeled Eppendorf tube and immediately stored at -80°C. This larvae collection method allowed collections of 5 larvae per stage per replicate without needing 25 larvae evenly distributed across the larval stages at one time to obtain a biological replicate. The process of collecting 5 larvae for each of the five larval stages was repeated until 3 biological replicates of each larval stage was collected and properly stored at -80°C.

Pupae were also collected from sex-specific mass mating jars. Similar to larvae, approximately 25 pupae were needed to obtain 25 mg of tissue. Female and male pupae were kept separate and coarsely assigned to early pupal stage,

mid pupal stage, late pupal stage based on physical attributes. In order to maintain a representative RNA sample of mixed stage pupae, 25 pupae from each of the three pupal stages were randomly and evenly selected and placed in an appropriately labeled Eppendorf tube. The single biological replicate, the Eppendorf tube containing pupae, was stored at -80°C. This process was repeated until three biological replicates for each sex were collected.

Adults were collected from vials that were maintained in a 21°C incubator located in the laboratory. The mass of a single female adult was determined to be approximately twice that of a single male adult. As a result, approximately 50 adult males were needed to obtain 25 mg of tissue, while approximately 25 adult females were needed to obtain the same amount of tissue. In a sterile work environment and using a CO_2 pad, female and male adults were collected separately in appropriately labeled Eppendorf tubes and stored at -80°C.

2.7 TRIzol total RNA extraction of sex-specific tissue across lifecycle

Following the collection of sex-specific tissue samples from stages across the *Sciara* life cycle, total RNA was isolated using the TRIzol reagent protocol. Due to differences in the storage of a complete biological replicate between larvae and embryos, and pupae and adults, the initial addition of TRIzol reagent and homogenization was handled slightly differently depending on the life cycle stage of the sample. The amount of embryos collected for each time lay differed. As 25 mg of tissue from the same sex and time lay became available, TRIzol was added (10% of the tissue mass), followed by homogenization by a sterile blue pestle. For mixed stage larvae, 5 larvae were coarsely staged and stored across five different larval stages. As a result, each larval sample was homogenized separately in 25 µl of TRIzol, using a sterile blue pestle. One homogenate from each of the five different larval stages was combined into an Eppendorf tube to produce a one complete mixed stage larvae biological replicate. This method maintained the ratio of TRIzol reagent to tissue outlined by ThermoFisher Scientific for a final TRIzol volume of 125 µl. For both pupae and adults, complete biological replicates were stored in the same Eppendorf tubes. Under

the fume hood, 150 µl of TRIzol were immediately added to these replicates upon removal from the -80°C freezer. Using a sterile blue pestle, the tissue was homogenized thoroughly. Following homogenization, total RNA from all tissue types was extracted (250 µl chloroform, 250 µl isopropanol, 500 µl 75% ethanol), and stored at -80°C as an RNA pellet. Prior to cDNA synthesis, the RNA pellet was taken through the remainder of the TRIzol protocol, including additional 75% ethanol washes and re-suspension in ultrapure water. The extracted RNA was quantified using Nanodrop (RNA-40 setting). The unused RNA was stored in 25 µl aliquots at -20°C.

2.8 Preparing female salivary glands across larval eyespot stages

Female larvae were immobilized in a generous drop of Robert's CR buffer, and precisely staged using a Zeiss phase contrast microscope. Serial dissections consisting of 5 salivary glands each were performed using sterilized forceps in droplets of Robert's CR buffer. Upon collection of 15 pairs of salivary glands per eyespot stage, the salivary gland tissue was taken through the TRIzol protocol. The resulting RNA pellet was suspended in 75% EtOH and stored at -20°C for downstream use. When needed, the RNA pellet was taken through the remainder of the TRIzol protocol and quantified using Nanodrop (RNA-40 setting). The remaining RNA was stored in 25 µl aliquots at -20°C.

2.9 Literature search and qPCR candidate reference gene selection

Potential reference genes were selected through a primary literature search using the Google Scholar search engine and searching relevant key words ("gene expression," "real-time quantitative PCR," "reference gene," "insect"). Reference genes with cellular maintenance and "housekeeping" functions used in other insect models for qPCR were chosen from the literature. Using FlyBase, the transcripts for selected reference genes were obtained for *Drosophila melanogaster*.

2.10 Designing and preparing candidate reference gene primers

Through basic genomics, the *D. melanogaster* transcripts were blasted against the *Sciara* salivary gland transcriptome and *Sciara* general transcriptome. The resulting *Sciara* transcripts were reverse blasted against the Diptera database in order to confirm that the extracted *Sciara* transcript encoded the desired reference gene in other Dipteran species. Transcripts that passed were used to design reference gene primers that span exon junctions using NCBI Primer Blast. Using an IGV browser, chosen primers were separately mapped to the *Sciara* salivary gland and general transcriptomes to ensure exon junction spanning when possible. It is important to note that similarities in exon junctions between different isoforms of the same reference gene led to isoform-specific primers that did not span exon junctions. Instead, these primers were designed within an isoform-specific exon. Potential primers were analyzed through primer pair analysis programs designed by John Urban, Ph.D. for specificity. Primer pairs that passed these specificity tests and met other imposed criteria (Tm, % GC, self-3' complementarity, etc.) were ordered from ThermoFisher Scientific. The primers were re-suspended in the appropriate volume of ultrapure water and stored at -20°C as 100 mM or 10 mM solutions.

2.11 DNA-free DNase treatment of RNA and cDNA synthesis

Using the Ambion DNA-free DNase treatment kit, contaminating DNA was removed from extracted RNA. The RNA was diluted to 10 ug in a 50 ul reaction and treated with 5 µl 10X DNase I Buffer followed by 1µl rDNase I. The reaction was incubated in a ThermoMixer heat block set to 37°C for 25 min. DNase was spun out of the reaction solution using 5 µl DNase Inactivation Reagent. RNA in the supernatant was carefully transferred to a new tube, quantified using Nanodrop (RNA-40 setting) and stored at -20°C for later use.

Total RNA and cDNA synthesis reagents stored at -20°C were thawed on ice. Using Nanodrop (RNA-40 setting) results, the RNA was diluted to 200-500 ng. The first strand synthesis reaction was prepared in a PCR tube (200-500 ng RNA in 11 µl, 1 µl random hexamers, 1 µl 10 mM dNTP) and incubated at 65°C

for 5 min. During this incubation, the second cDNA synthesis mix (4 µl 5X FSS reaction buffer, 1 µl 0.1M DTT, 1 µl murine RNase inhibitor, 1 µl SSIII RT) was prepared. Both reaction mixes were spun down, shortly incubated on ice and combined. The "FSS" program set up by John Urban, Ph.D. was run (25°C, 10 min.; 50°C, 50 min.; 85°C, 5 min.; 4°C, forever).

2.12 Validating Sciara *candidate reference gene primers*

The working protocol established for genomic DNA for DNA puff qPCR served as the framework for cDNA primer validation. Using Qubit reagents, cDNA was quantified and diluted to 1 ng/µl. A serial dilution was performed from the 1:1 cDNA stock (1 ng/µl) to create 1:4, 1:16 and 1:64 cDNA solutions. Primer master mixes for prepared for each primer pair. Well maps for 96-well plates were designed in Microsoft PowerPoint and carried out with primer master mixes added column-by-column and cDNA solutions added row-by-row. The plate was spun down using the centrifuge in the Larschan Lab, and qPCR was run on an Applied Biosystems 7300 Real-Time PCR system with added dissociation curves. The results of each run were analyzed in RStudio for primer pair specificity and reaction efficiency.

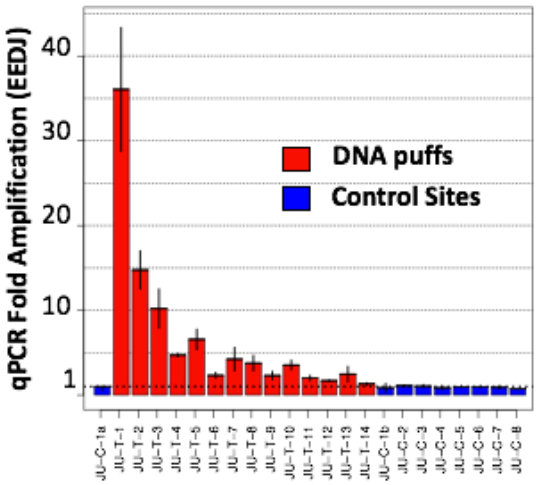
Results

3.1 qPCR of DNA Puffs

To become familiar with qPCR methodology and techniques, qPCR of DNA puffs was performed. Primer validation was performed using primers designed for control loci that fail to undergo DNA amplification and target loci (candidate DNA puffs) with high copy number derived from Dr. John Urban's sequencing data and genome assembly. Using salivary gland DNA obtained from edge-eye/drop jaw (EEDJ) female larvae and genomic DNA obtained from female adults, the relative fold amplification of potential DNA puff sites was determined. The experiments were performed in biological and technical triplicate. In contrast to previous data demonstrating that DNA amplification plateaus following the 14x7 eyespot stage, results from these experiments clearly

illustrate that DNA amplification continues into the EEDJ eyespot stage for select DNA puffs. Compared to the control sites that expectedly underwent a fold enrichment of 1 (i.e. no amplification), the target sites underwent DNA amplification. Included in these target sites were DNA puffs II/9A (JU-T-1) and II/2B (JU-T-2), which served as positive controls for each test plate (Figure 8). These results confirm sequencing data and served as important practice for future qPCR experiments.

A



B

Primer Pair	Fold Enrichment
JU-C-1a	1.00
JU-T-1	33.3
JU-T-2	12.9
JU-T-3	8.11
JU-T-4	4.55
JU-T-5	5.23
JU-T-6	2.04
JU-T-7	3.46
JU-T-8	2.76
JU-T-9	1.79
JU-T-10	3.43
JU-T-11	2.25
JU-T-12	2.64
JU-T-13	1.94
JU-T-14	1.25
JU-C-1b	0.98
JU-C-2	1.05
JU-C-3	1.06
JU-C-4	0.85
JU-C-5	0.95
JU-C-6	0.86
JU-C-7	0.76
JU-C-8	0.84

Figure 8. qPCR of candidate DNA puffs performed on EEDJ female salivary glands. (A) Amplification at DNA puffs II/9A (JU-T-1) and II/2B (JU-T-2) continues through the EEDJ eyespot stage. (B) Fold enrichment values for control loci and target loci.

3.2 Testing different qPCR recipes

qPCR remains the gold standard for investigating specific changes in gene expression due to its high sensitivity. However, the high cost of qPCR reagents renders this method undesirable for high throughput purposes. In order to reduce costs, the original 15 µl recipe was proportionately reduced to a 10 µl qPCR recipe and tested. Using three control primers and three target primers, volumes of qPCR primer master mixes were adjusted for both primer validation and test plate protocols. The DNA volume in both protocols was maintained at 2 µl (50 pg/µl). RStudio analyses demonstrated that the previously validated primer pairs also passed primer validation using a 10 µl recipe. After these primers passed validation, the 10 µl recipe was used to perform DNA puff qPCR on EEDJ female salivary glands. Test plates were performed using three control sites and three target sites. The results matched the outcome observed using the original 15 µl recipe. DNA amplification during the EEDJ stages was seen at the three target sites tested, including DNA puffs II/9A and II/2B. The three control sites tested had an expected fold enrichment of 1. In order to ensure reproducibility and reliability, primer validation plates and test plates were performed in biological triplicate using genomic DNA from EEDJ female salivary glands.

In an effort to further reduce reagent costs, a 5 µl qPCR recipe was tested. Using the same control and target sites, the volumes of the qPCR reagents were proportionally adjusted and the DNA volume was reduced to 1 µl (50 pg/µl). The same three control and target primers were used to test the 5 µl primer validation recipe, and all six primers passed. The results obtained from using the 5 µl recipe to determine relative fold enrichment using a qPCR test plate were unreliable and different from the expected results. Even though EEDJ salivary gland DNA was used to test the 5 µl recipe, the fold enrichment at DNA puff II/9A was approximately half of what had been shown in previous qPCR experiments. obtained from the original 15 µl recipe and adjusted 10 µl recipe. Additionally, the fold enrichment observed at the two other target sites were substantially less than expected.

Moving forward, the 10 µl recipe for both primer validation and test plates were used for subsequent qPCR experiments. This adjusted recipe effectively reduces qPCR resources by approximately 33%, which is important for the many qPCR experiments that will be carried out to determine differences in gene expression levels across the eyespot larval stages and the *Sciara* lifecycle.

3.4 Practice TRIzol extraction of whole tissue and salivary glands

Preliminary TRIzol extraction experiments were performed in order to gauge the amount of tissue needed for high quantity and quality RNA. Approximately 38 mg of *Sciara* female mixed stage pupae stored at -80°C was used for the practice TRIzol total RNA extraction. RNA was initially quantified using Nanodrop (RNA-40 setting). The purity of the RNA sample was assessed by the 280/260 and 280/230 Nanodrop readings. Qubit RNA reagents were used in an attempt to further quantify RNA, but the reagents were not working properly. Because the Nanodrop readings were consistent with pure RNA, the Nanodrop concentration obtained (2488.9 ng/µl) was used to determine future dilutions. Gel electrophoresis was carried out to confirm the integrity of the RNA extracted. High integrity RNA should yield two crisp rRNA bands between 1kb and 4kb along with a tRNA smear at the bottom of the gel. The extracted RNA yielded two bands and substantial tRNA (Figure 9). These results suggest that 38 mg of tissue is more than enough material to yield high concentrations of high integrity RNA.

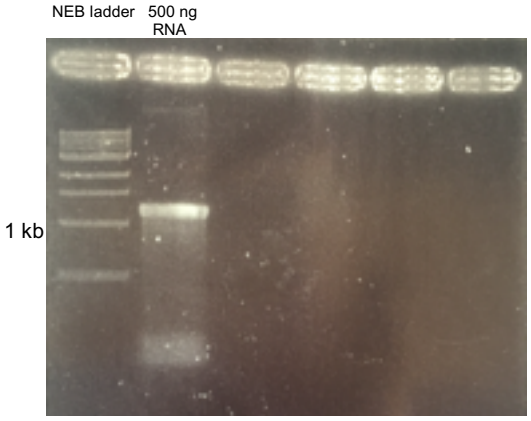


Figure 9. Gel electrophoresis of total RNA extracted from female mixed stage pupae. Lane 1 contains 10 ul NEB 1 kb DNA ladder. Lane 2 contains a 500 ng RNA sample extracted from mixed stage female pupae.

Additionally, a preliminary TRIzol experiment was performed on salivary glands dissected from female larvae in the ≤ 8x4 eyespot stage to determine the quantity and quality of RNA extracted. Because DNA obtained from 15 pairs of salivary glands were successfully used for qPCR of DNA puffs, 15 pairs of salivary glands was tested for RNA yield and integrity. Gel electrophoresis revealed sharp rRNA bands and a tRNA smear at the bottom of the gel, confirming high integrity RNA (Figure 10). The Nanodrop reading obtained for these 15 pairs of salivary glands was 585.2 ng/µl. Approximately 2 ng of cDNA is needed per reaction in a 96-well plate. A full qPCR plate requires approximately 192 ng cDNA. As such, 15 pairs of salivary glands yield more than enough high quality RNA for a complete qPCR experiment.

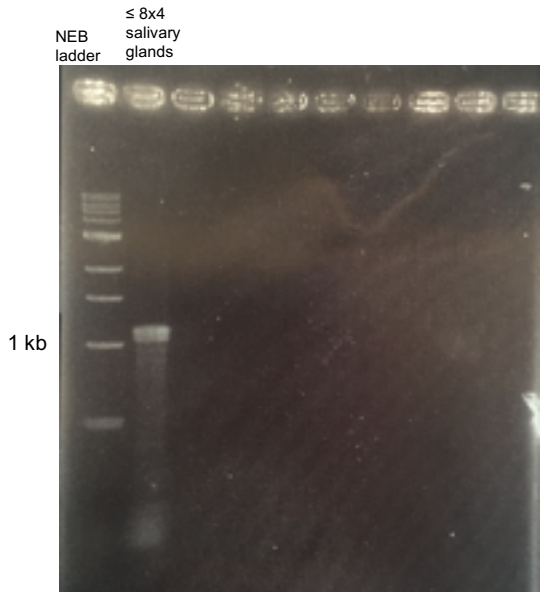


Figure 10. Gel electrophoresis of total RNA extracted from 15 pairs of ≤ 8x4 female salivary glands. The arrow indicates the 1kb ladder marker. Lane 1 contains 10 µl NEB 1 kb DNA ladder. Lane 2 contains a 500 ng RNA sample extracted from ≤ 8x4 eyespot larvae.

3.4 Average mass of larvae, pupae and adults

The TRIzol manufacturer's protocol uses 1 mL TRIzol per 50 mg of tissue. In order to maintain the proper TRIzol to tissue ratios outlined in the manufacturer's guidelines, 25 mg of sex-specific tissue from each *Sciara* lifecycle stage was collected and stored as RNA at -20°C for future use. Before sample collection, the average mass of *Sciara* larvae, pupae and adults was measured to ensure tissue collection as close to 25 mg as possible. Because *Sciara* female adults and male adults substantially differ in mass, the average mass of female adults and male adults were determined separately. For each lifecycle stage, 25 specimen were collected into a pre-weighed Eppendorf tube. The total mass was divided by 25, and the average mass of an individual larva, pupa, female adult and male adult was determined. The average masses for a single specimen across the *Sciara* lifecycle stages are as follows:

***Sciara* lifecycle stage**	**Mass per single specimen (mg)**
Male and female larvae	2
Male and female pupae	0.9 to 1
Male adults	0.3
Female adults	0.5

3.5 Selection of candidate reference genes

To accurately assess differential gene expression using reverse transcription qPCR, at least one stable reference gene needs to be used. Fundamentally, potential reference genes should yield constant expression levels at different stages of development and should also have expression levels above background. Ultimately, the expression level of the genes of interest will be normalized to the reference gene, so it is extremely important that the reference gene is consistently, reliably and stably expressed. Unfortunately, the *Sciara* community does not currently have reliable reference genes available. In order to find such control genes in *Sciara*, a literature search was performed. Potential reference genes were identified as genes that were successfully used in reverse transcription qPCR experiments in other insect systems (Table 3).

Primary paper	Reference genes used
Validation of reference genes for expression analysis by quantitative real-time PCR in *Leptinotarsa decemlineata* (Shi et al., 2013)	Actin, ADP-ribosylation factor, TATA-binding protein (TBP), ribosomal protein, translation elongation factor
Validation of reference genes for gene expression studies in the honey bee, *Apis mellifera*, by quantitative real-time RT-PCR (Lourenco, et al., 2008)	Actin, ribosomal protein, elongation factor, TBP-associated factor
Validation of reference genes for quantitative expression analysis by real-time RT-PCR in four lepidopteran insects (Teng et al., 2012)	Actin, GAPDH, elongation factor, ribosomal protein
Reference gene selection for insect expression studies using quantitative real-time PCR: The head of the honeybee, *Apis mellifera*, after a bacterial challenge (Scharlaken et al., 2008)	GAPDH, actin, ribosomal protein
Looking for reference genes for real-time quantitative PCR experiments in *Rhodnius prolixus* (Majerowicz, et al., 2011)	Ribosomal protein, elongation factor
Validation of reference genes for expression analysis in the salivary	18S rRNA, GAPDH, actin, tubulin, ribosomal protein

gland and the intestine of *Rhodnius prolixus* under different experimental conditions by quantitative real-time PCR (Paim et al., 2012)	

Table 3. Results from the candidate reference gene literature search.

In general, the candidate reference genes chosen have cellular housekeeping functions (Table 4). These genes are required for the maintenance of basic cellular function and are typically constituitively expressed in all cells of an organism. Due to their role in cellular upkeep and uniform expression with low variance, these genes serve as excellent candidate reference genes and potential normalizers for qPCR experiments.

Symbol	Gene name	Function
18SR	18S ribosomal RNA	Structural RNA for eukaryotic ribosomal subunit
ACT	Actin	Cytoskeletal structural protein involved in cell motility
ARF	ADP-ribosylation factor	GTP-binding proteins involved in protein trafficking
EF	Elongation factor	Facilitate translational elongation during protein synthesis
GAPD	Glyceraldehyde 3-phosphate dehydrogenase	Catalyzes reversible oxidative phosphorylation of glyceraldehyde-3-phosphate
TBP	TATA-binding protein	Transcription factor involved in initiating RNA synthesis
RP	Ribosomal protein	Encodes a ribosomal subunit protein
TUB	Tubulin	Cytoskeletal structural protein

Table 4. Candidate reference genes evaluated and their functions.

3.6 Primer pair design for selected candidate reference genes

A large part of this project required an understanding of basic genomics and primer design. Because *Sciara* does not currently have its own database of genes and genomes, *Drosophila* transcripts for candidate reference genes were pulled from FlyBase. The *Drosophila* transcripts were blasted against the recently available *Sciara* salivary gland transcriptome and general transcriptome. *Sciara* transcripts with high sequence similarity to the *Drosophila* transcripts were pulled from the transcriptome data. In order to ensure that the extracted *Sciara* transcripts corresponded to the correct reference gene, the *Sciara* transcripts were reverse blasted against a Dipteran gene database. The *Sciara* transcripts

that correctly matched the intended reference genes were used to design primers.

Primers were designed for candidate reference gene transcripts extracted from the *Sciara* transcriptomes (Table 5). Primer pair analyses were performed using RStudio scripts designed by John Urban, Ph.D. If primer pairs failed specificity tests, then NCBI Primer Blast settings were altered with less stringent parameters. The final primers were designed to meet the following criteria:

Tm = 57°C to 66°C
Max Tm difference = 1°C
Length = 18 – 26 bp (per primer)
% GC content = 43 – 69%
Self-complementarity ≤ 5
Self-3'-complementarity ≤ 2

Symbol	Forward primer	Reverse primer
18SR	AAAGGAATTGACGGAAGGGC	AAATTAAGCCGCAGGCTCC
ACT	CGGTGGTTCCATCCTTGCT	GCACTTGCGGTGGACAATTC
ARF	ATCGCGATACAGCAATATAGCATCA	AGCTAAATTCGTTACTTGCGAGC
EF	GCTGATTTGAGATCGAATACTGGC	GATCACCTGGGAATACCTGCC
GAPD	TGGAAATCGTTGTGAACAGTTGTG	ACGACCAATACGGCCAAATCC
TBP	ATTGCTCTACATGCGAGAAATGC	GGCAGTTGTTCGTGGTTCTCT
RP	TCGGAACGCTGAACATGGAT	GCTTCGGACGCTGTTTGTTC
TUB	AACCCAATTCAAAATGCGTGAATG	CAACTCCCAGCAAGCGTTTC

Table 5. Primer sequences for candidate reference genes

Additionally, most primers were designed to span predicted intron-exon boundaries in order to maintain specificity to transcript cDNA over contaminating genomic DNA. Primer pairs were aligned to the salivary gland transcriptome and general transcriptome using the Integrative Genomics Viewer (IGV) (Figure 11). Genomic DNA contains introns, while cDNA does not. Primers that span exon junctions will either fail to properly anneal to or amplify contaminating genomic DNA. In contrast, such primers will selectively and effectively amplify cDNA template. Throughout the primer design process, the primer specificity analyses revealed multiple sequences along the transcriptomes that were complementary to the primer sequences. Further investigation uncovered that the primer pairs were complementary to candidate reference gene isoforms. To increase primer

specificity to a single isoform, all isoform transcripts for each candidate reference gene were extracted from the *Sciara* transcriptomes. The intron-exon boundaries of isoforms for a particular gene were aligned in IGV in an attempt to identify isoform-specific exon junctions. When possible, primers were designed to span isoform-specific exon junctions in order to ensure that only one particular gene was being amplified at a time. In some cases, candidate reference gene isoforms shared the same predicted intron-exon boundaries. For these genes, primers were designed within a region of isoform-specific exons to ensure that only one isoform for a particular reference gene was being amplified at a particular time.

Figure 11. Examples of primer pairs (bottom of each image) spanning predicted intron-exon boundaries of the *Sciara* general transcriptome (top of each image) and salivary gland transcriptome (middle of each image).

3.7 Testing genomic DNA contamination of RNA

Because some primers were designed within exons, contaminating genomic DNA needed to be removed from extracted RNA before cDNA synthesis. A DNA-free DNase treatment kit, recommended by Leo Kadota, B.S., was tested to ensure DNase inactivation following the DNase treatment. RNA samples extracted from mixed stage female larvae was split between a DNase inactivation reaction and a mock DNase inactivation reaction. The DNase

treatment reaction was followed as outlined in the manufacturer's protocol. The final step of the reaction involves spinning out the DNase through a DNase inactivation reagent. The DNase inactivation reagent was not spun out in the mock reaction. *Sciara* genomic DNA was added to both RNA samples and the solutions were analyzed electrophoretically. As a positive control, adult genomic DNA was loaded onto the gel. The results of this control experiment illustrate that the DNase inactivation reagent reliably removes DNase from the reaction, as evidenced by the presence of genomic DNA in the DNase inactivation reaction. In contrast, genomic DNA is absent from the solution in which the DNase inactivation reagent was not removed (Figure 12).

Additionally, small-scale qPCR experiments were performed to determine whether or not RNA treatment with the DNase kit altered Ct values. RNA was prepared from female mixed stage larvae using TRIzol. Extracted RNA was split into three different conditions: no DNase, DNase and mock DNase treatments. The "no DNase" RNA sample was synthesized into cDNA without intermediate DNA removal. The "DNase" and "mock DNase" RNA samples went through the DNA-free DNase treatment procedure before cDNA synthesis. DNA-free, RNA-free water was used in place of DNase for the "mock DNase" RNA sample. The three different RNA samples were synthesized into cDNA and qPCR was used to identify changed in Ct values. The qPCR results illustrated that DNase treatment before cDNA synthesis did not affect the amount of cDNA template in the reaction sample as the Ct values were comparable throughout all three RNA treatment conditions. Moving forward, DNase treatment was used for experimental samples in order to mitigate the effects of contaminating genomic DNA.

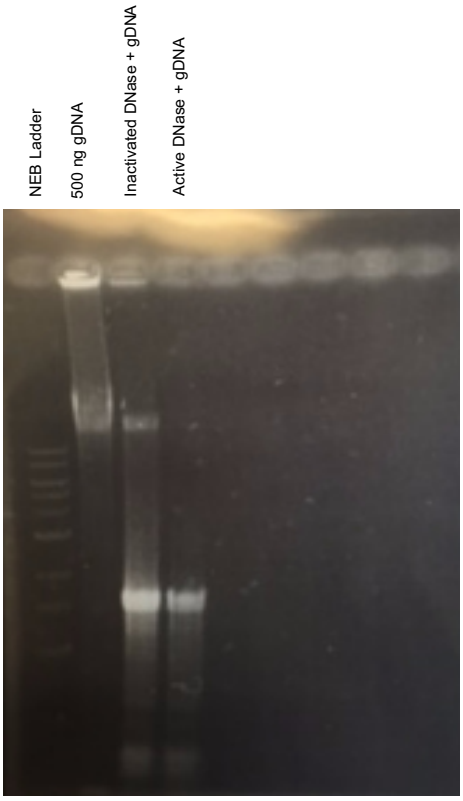


Figure 12. Gel electrophoresis of total RNA extracted from female mixed stage larvae after DNase inactivation and mock DNase inactivation. Lane 1 contains 10 µl NEB 1 kb DNA ladder. Lane 2 contains 500 ng adult genomic DNA. Lane 3 contains RNA and inactivated DNase with exogenous adult genomic DNA. Lane 4 contains RNA and active DNase with exogenous adult genomic DNA.

3.8 cDNA primer validation using staged salivary glands

Before performing full plate qPCR experiments using cDNA, many small plate experiments were performed in order develop a working protocol for cDNA samples. Using an established qPCR primer validation protocol, candidate reference gene primer pairs were validated using cDNA synthesized from staged salivary glands. The qPCR results were analyzed in RStudio scripts created by John Urban, Ph.D. designed to evaluate primer pairs based on specificity and reaction efficiency. The candidate reference gene primer pairs that passed validation were actin, elongation factor, ribosomal protein, GAPDH, tubulin. The candidate reference gene primers that failed validation were 18S rRNA, TATA-binding protein and ADP-ribosylation factor (Table 6). The failed primer pairs either had low reaction efficiency or formed primer dimers during the PCR cycles based on the dissociation curves.

Pass	Fail
Actin	
Elongation factor	18S rRNA
Ribosomal protein	TATA-binding protein
GAPDH	ADP-ribosylation factor
Tubulin	

Table 6. Candidate reference gene primer pairs that passed primer validation on staged salivary gland cDNA.

Further qPCR experiments indicate that the expression levels of the candidate reference genes changes across larval development. A preliminary experiment showed differences in Ct values between salivary gland cDNA prepared from female ≤ 8x4 larvae and female EEDJ. These results potentially indicate that expression levels of candidate reference genes do differ across development. Additional work will need to be performed in order to determine the extent of these differences in raw data and whether or not these differences persist across the lifecycle stages.

Discussion and Future Directions

Sciara undergo developmentally regulated DNA amplification and chromosome elimination, which make this organism a valuable system for studying basic mechanisms involved in developmental and molecular biology. The genome and transcriptome resources that have recently become available serve as valuable tools to further understand *Sciara* as a model organism. Differences in gene expression levels throughout larval development and over the lifecycle may partly explain genes responsible for DNA amplification/DNA puff proteins and chromosome elimination/sex determination, respectively. In order to validate transcripts of interest mined from the salivary gland transcriptome and general transcriptome, at least one stable reference gene is required. However, recent Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines outline that at least two reference genes should

be used when performing RT-qPCR to quantify relative gene expression (Bustin et al., 2009). Currently, the *Sciara* community lacks such reference genes.

These preliminary results lay the foundation for subsequent work to identify reference genes that will serve as normalizers for genes of interest. This research establishes experimental procedures and conditions for future endeavors that require normalizing control genes.

Further experiments will be performed in order to elucidate the degree to which Ct values differ for the candidate reference genes across larval development and the lifecycle stages. Software-based approaches such as geNorm, NormFinder and BestKeeper, serve as suitable tools to validate variation across developmental time in order to comparably and statistically quantify reference gene expression levels. The validated transcripts of interest will provide insight into the mysterious nature of *Sciara* biology and development, placing this organism in a new realm of scientific research. Our deeper understanding of *Sciara* translates into a deeper understanding of cellular and molecular biology as it occurs in humans.

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Appendix: Materials and Methods

Collecting salivary glands for DNA puff qPCR

Staging larvae

1. Pick and plate female larvae, and coarsely re-assign larvae to 4-5 other plates--no eye spots, early, mid, late, edge eye/drop jaw
2. Further stage larvae of interest using compound scope
    a. Place 2 slides on metal ice block, and 1 mL of Robert's buffer CR on ice
    b. Place agar plate containing coarsely assigned larvae on ice
    c. Add 2 separate drops of 40-60 µL of Robert's buffer to slide, and then place one larva on each puddle of buffer with eyespots facing up
    d. Gently place coverslip on both larvae, and place slide on ice block
    e. Repeat Steps c-d using second slide, and other larvae, totaling 4 larvae on 2 slides
    f. Remove first slide from ice block, and quickly wipe bottom of the slide
    g. Position left larva under light beam of compound scope, and record exact eyespot stage
    h. Continue to the next larva on same slide
    i. Remove coverslips, and place larvae onto appropriately labeled plates
    j. Put 2 new larvae on slide, and place coverslip on top
    k. Place slide on ice block, and process second slide
3. Place staged larvae on separate plate of given stage
4. Begin dissections immediately (within 2 hours of staging)

Salivary gland dissections for DNA puff qPCR– 15 pairs of salivary glands per biological replicate

1. Place 100 µL of DNAzol in microtube, and keep on ice tray
2. Aliquot 1.5 mL of Robert's buffer CR into separate microtube, and place on ice tray
3. Aliquot 250-300 µL of cold Robert's buffer CR onto slide to form a horizontally elongated puddle
4. Place 5, precisely staged female larvae, evenly spaced, across puddle
5. Starting with larva on the left, remove salivary glands from larval body
6. Clean each pair of salivary glands, removing excess tissue and fat bodies
7. Collect 5 pairs of salivary glands, and shake them
8. Repeat steps 2 through 7, placing dissected salivary glands in same DNAzol microtube for a total of 15 pairs of salivary glands per biological replicate

DNAzol genomic DNA isolation and quantification for DNA puff qPCR

Notes:

- Use at most 10 µg RNase (2.5 µL at 4 mg/µL OR 1 µg/µL) in 1 mL DNAzol
- Adjust volume of RNase A and Proteinase-K when using smaller volumes of DNAzol

Genomic DNA isolation of adult fly DNA using DNAzol

1. Turn heat block to 37°C
2. Measure weight of adult flies by massing an empty 1.5 mL Eppendorf tube, then filling ⅓-½ of tube with flies and measuring the mass of the tube
    a. The difference with original mass is the mass of flies
3. For adult fly DNA: Grind Sciara specimen using one of two methods
    a. Method 1:
        i. Add 100-200 µL DNAzol to 25-50 mg Sciara tissue in Eppendorf tube
        ii. Pushing and twisting specimen with sterile blue pestle, thoroughly grind tissue
        iii. Add remaining 800-900 µL DNAzol for a total of 1 mL DNAzol
    b. Method 2: Snap freeze
        i. Pour liquid nitrogen in bowl and place Eppendorf tube containing specimen in bowl
        ii. Once the bubbling has subsided, remove Eppendorf tube and grind specimen using sterile blue pestle
        iii. Dunk Eppendorf tube in bowl of liquid nitrogen and continue grinding specimen until it is a fine powder
        iv. Add 100-200 µL DNAzol to Eppendorf tube, and continue grinding
        v. Add remaining 800-900 µL DNAzol, using DNAzol to rinse tissue stuck to pestle
4. Invert Eppendorf tube 10x to mix contents
5. Add 5 µL RNase, and incubate at 37°C for 10 min.
6. Add 5 µL Proteinase-K, and incubate at 37°C for 10 min.
7. Spin at 10,000 g for 10 min. to remove large debris
8. Transfer viscous supernatant, which contains DNA, to new 1.5 mL Eppendorf tube
9. Add 500 µL 100% molecular grade EtOH per 1 mL DNAzol reagent (i.e. ½ volume)
10. Slowly invert Eppendorf tube 40-50x (cloudiness/white precipitate should form)
11. Incubate at RT for 2 min., then incubate on ice for 2 min.
12. Spin at 4,000 g for 2 min to form DNA pellet
13. Remove supernatant (keep pellet), and repeat wash steps one to two more times if necessary

    a. Potential break point: DNA pellet can be stored at this step in 70-80% molecular grade EtOH at -80°C
14. Add 0.8-1 mL 75-80% molecular grade EtOH to tube containing DNA pellet
15. Slowly invert 3-6x and flick to dislodge pellet
16. Spin at 4,000 g for 10 min. to form DNA pellet
17. Remove EtOH from pellet by pipetting or decanting
18. Repeat 75-80% EtOH wash (Steps 15-18) for a total of 2-3 washes
19. After final EtOH wash, air dry DNA by leaving tube open for ~30 seconds and making sure that the DNA remains moist/glistening
20. Re-suspend DNA in 100-400 µL 1X TE (Tris + EDTA buffer) or 10 mM Tris-HCl (both pH 7.5-9.0) (Note: Clean DNA before use if re-suspended in TE)
21. Incubate at 37C for at least 1 hr., then incubate at RT overnight
22. Spin down and store at 4°C

Genomic DNA isolation of salivary gland DNA using DNAzol

1. Dissect staged larvae in Robert's CR buffer and collect salivary glands in 100 µL DNAzol on ice
2. Using sterile blue pestle, stroke salivary glands in DNAzol 5-10X
3. Wash blue pestle with another 100 µL DNAzol into the tube (200 µL DNAzol total)
4. Add 2 µL RNase
5. Incubate tube at 37°C for 10 min.
6. Add 2 µL Proteinase-K
7. Incubate tube at 37°C for 10 min.
8. Add 100 µL 100% EtOH (½ volume of DNAzol)
9. Slowly invert tube several times to mix, allowing DNA to precipitate out
10. Pellet DNA by centrifuging sample at full speed for 10 min.
11. Carefully remove supernatant, and re-suspend DNA pellet in 1 mL of 70% EtOH
    a. Potential break point: DNA pellet can be stored in 70% molecular grade EtOH at -80°C
12. Slowly invert 3-6x and flick to dislodge pellet
13. Spin at 4,000 g for 10 min. to form DNA pellet
14. Remove EtOH from pellet by pipetting or decanting
15. Repeat 75-80% EtOH wash (Steps 15-18) for a total of 2-3 washes
16. After final EtOH wash, air dry DNA by leaving tube open for ~30 seconds and making sure that the DNA remains moist/glistening
17. Re-suspend DNA in 100-400 µL 1X TE (Tris + EDTA buffer) or 10 mM Tris-HCl (both pH 7.5-9.0) (Note: Clean DNA before use if re-suspended in TE)
18. Incubate at 37°C for at least 1 hr., then incubate at RT overnight
19. Spin down and store at 4°C

Cleaning genomic DNA using AMPure Beads

1. Allow AMPure beads to reach room temperature, then vortex beads to resuspend them
2. Label three 1.5 mL Eppendorf tubes: “starting DNA,” “AMP sup,” and “AMP clean DNA”
3. Pipet 50 µL DNA into “starting DNA” tube, then add 50 µL UPW (total volume = 100 µL)
4. Pipet 100 µL AMPure bead suspension into “starting DNA” tube (total volume = 200 µL)
5. Mix contents of “starting DNA” tube by gently pipetting up and down, or flicking tube
6. Briefly spin “starting DNA” tube using mini centrifuge to collect contents at bottom
7. Incubate tube at room temperature for 15 min., then transfer tube to magnetic stand and incubate for another 10 min.
8. Keep the “starting DNA” tube on magnetic stand, and carefully pipet supernatant (~200µL) and place in “AMP sup” tube
9. While “starting DNA” tube is still on magnetic stand, add 500 µL of 70-80% molecular grade EtOH to tube, then incubate at room temperature for 30s
10. Carefully remove supernatant from “starting DNA” tube and dispose
11. Repeat the EtOH wash using 200 µL, then briefly spin the tube in mini centrifuge
12. Place “starting DNA” tube back on magnetic stand for 30 s, then remove excess EtOH using P20
13. Air dry beads on magnetic stand (lid of Eppendorf tube open) for 15 min.
14. Elute DNA off beads into desired volume/solvent for downstream applications
15. Mix contents (beads/eluted DNA) thoroughly by pipetting up and down, or flicking
16. Briefly spin tube in mini centrifuge, then incubate tube at 37C for 20 min. using Thermomixer (no mixing)
17. Place tube on magnetic stand for 5 min., then pipet/transfer supernatant to “AMP clean DNA” tube
18. Obtain Nanodrop and Qubit measurements, then run a confirmation gel
19. Store at 4°C

Quantify genomic DNA (Nanodrop)

1. Prepare Eppendorf tube of UPW and another tube of buffer that DNA is suspended in (1X TE or Tris-HCl)
2. Open Nanodrop software and select “Nucleic Acids”
3. Write in Sample ID, then clean device with UPW and Kimwipes
4. Pipet 2 µL UPW onto designated surface, then close surface arm
5. Press “OK,” then clean device
6. Pipet 2 µL buffer onto cleaned designated surface, then close surface arm
7. Press “Blank,” then clean device

8. Pipet 2 µL sample onto designated surface, then press “Measure”
9. Record the following data:
    a. A260/280 > 1.8 is good and routinely obtained (measures protein contamination; absorbance at 280 nm mostly from tryptophan)
    b. A260/230 > 2.0 is good, but never obtained before cleaning (the 230 nm peak is due to guanidinium salts, carbohydrates, other DNAzol components; this peak disappears after cleaning)
    c. Concentration of DNA (in ng/µl) (very inaccurate when DNA is dirty; can be overestimated by up to 10x)
10. Print results (optional) and clean device using UPW and Kimwipes

Quantify genomic DNA (Qubit)

1. Prepare reaction tubes for 2 standard curves and # of samples being analyzed (i.e. analyzing x samples requires x+2 tubes)
2. Prepare working solution by adding 199 µl “dsDNA Qubit buffer, high sensitivity” for each reaction prepared to a 1.5 ml Eppendorf tube (Note: Buffer and Qubit dye stored in the small drawer to the left of John Urban’s fridge)
3. Briefly spin Qubit dye (Qubit dsDNA HS reagent) in mini centrifuge, then add 1 µL dye for each reaction tube (x+2 µL dye; total volume of 200 µL for each reaction)
4. Vortex Eppendorf tube containing Qubit buffer and dye
5. Add 190 µL working solution to two separate Qubit tubes
6. Add 10 µL ‘high’ standard solution to one Qubit tube, and 10 µL ‘low’ standard solution to other Qubit tube (Note: Standard solutions are stored in small plastic bag in 4°C fridge)
7. Vortex tubes and let sit for 2 min.
8. Add 199 µL working solution to Qubit tube, then add 1 µL DNA sample
9. Pipet to mix (do not vortex)
10. To operate Qubit fluorometer (Larschan Lab): “dsDNA HS” > Go > New calibration
    a. Wipe tube containing Low standard solution with Kimwipe, then place in Qubit
    b. Remove Low standard solution, and wipe tube containing High standard solution
    c. Place High standard solution in Qubit, then proceed to Sample measurement
    d. Wipe tube containing Sample, then place in Qubit (Note: Do not hold temperature-sensitive tubes at base. Make sure there are no air bubbles in the solution.
    e. Choose “Calculate sample concentration,” then select appropriate volume (in µL) of sample added to Qubit tube (usually 1 µL)
11. Continue reading samples using Qubit and recording sample concentrations

DNA puff qPCR primer validation (10 ul recipe)

Notes:

- Check for starting materials at beginning of day (autoclaved Eppendorf tubes, female adult DNA, etc.)
- Sign up for qPCR machine for time expected to be done preparing qPCR plate

1. Set up 96-well map/matrix to include primer pairs that need qPCR validation
2. Make 1 µM primer solutions (-20°C freezer)
    a. Add 180 µL of UPW to 12 pre-labeled Eppendorf tubes
    b. Add 20 µL of corresponding 10 µM primer to each tube
    c. Thaw primers on ice
    d. Set up workbench (fill 3 ice buckets, empty waste container, wipe down surfaces with EtOH, etc.)
3. For gDNA dilutions, working concentration is 1 ng/µL and 2 ng will be used per reaction
    a. First, Qubit gDNA to obtain concentration
    b. Dilute gDNA to 1 ng/µL in sufficient volume for all reactions (24 reactions/plate) and dilution series to obtain 1:1 gDNA
        i. Need at least 48 µL at 2 µL per reaction; taking into consideration 24 reactions per plate, 25 µL for dilution, and pipetting error, make 100 µL at 1 ng/µL
    c. Make 1:4 by combining 25 µL 1:1 with 75 µL UPW
    d. Make 1:16 by combining 25 µL 1:4 with 75 µL UPW
    e. Make 1:64 by combining 25 µL 1:16 with 75 µL UPW
4. Prepare master mixes on ice— 8 reactions per primer pair; up to 12 primer pairs per plate; make enough master mix for 10 reactions
    a. Label 12 Eppendorf tubes with primer pair names
    b. Tape pipette to appropriate volume and follow the recipe below:

        9 reactions using 1 µM primers:
        9 µL UPW (add first)
        45 µL 2X SYBR Green
        9 µL of 1 µM forward primer
        9 µL of 1 µM reverse primer

    c. After adding final component of master mix, pipette up and down to mix
    d. Shortly spin master mixes down
    e. Place 1 µM primer stock solutions back in -20°C freezer
5. Load columns of plate with corresponding primer master mixes
    a. Place plate on ice in empty tip platform with barcode facing you
    b. Add primers left to right, one column at a time
    c. Add 8 µL of master mix in designated well according to well map
    d. Keep all plate columns to the right of current column covered with optical tape without exposing sticky part
    e. Only pipette over covered regions and current well

f. Drop master mix solution along side of well

6. Load rows with corresponding DNA concentrations (high to low)
    a. Add DNA top to bottom, one row at a time
        i. Rows A and B = 1:1 (1:1)
        ii. Rows C and D = 1:10 (1:4)
        iii. Rows E and F = 1:100 (1:16)
        iv. Rows G and H = 1:1000 (1:64)
    b. Add 2 µL of DNA to each well, dropping solution along side of well
7. Seal plate using sticky side of the optical tape
8. Spin plate down at 1000 RPM for 1 min. (Larschan Lab)
    a. Remove circular centrifuge chamber and insert plate chamber
    b. After spinning plate down, check for air bubbles
    c. Remove air bubbles by spinning plate again
9. Turn on computer and qPCR machine
10. Open the qPCR computer program
    a. Start new document
    b. Accept settings
    c. Add appropriate primer sets (Reporter dye = SYBR, quencher dye = none, passive reference dye = ROX)
    d. Highlight appropriate wells and label as particular primer set
    e. Within each primer set, label wells as either standard, NTC, or "unknown' according to what they are
    f. Add dissociation stage in 'Instrument' tab
    g. Set 'Sample Volume' to correct sample volume (i.e. 10-25 µL)
11. Gently wipe bottom of qPCR plate with KimWipe, then place plate in machine with barcode facing you
12. Start qPCR cycles
13. When qPCR is complete (~2.5 hours), go to 'Analysis' tab
    a. Take notes on amplification plots and dissociation curves
    b. Export results to thumb drive
14. Analyze results in Excel or R to determine whether or not each primer passes validation

<u>DNA puff qPCR test plate</u>

Notes:

- Check for starting materials at beginning of day (autoclaved Eppendorf tubes, female adult DNA, etc.)
- Sign up for qPCR machine for time expected to be done preparing qPCR plate

1. Set up 96-well map/matrix to include appropriate primer pair test sites
2. Make primer set master mixes for the number of total reactions for each primer set plus one

    7 reaction recipe using 1 µM primers:
    7 µL UPW
    35 µL 2X SYBR Green
    7 µL forward primer

7 µL reverse primer

3. Dilute genomic DNA for "unknown" calibrator adult fly DNA sample to 50 pg/µL (or 100 pg/µL) in total volume needed using Tris-HCl
    a. Need 2 µL of DNA per sample * 3 samples per test site = 6 µL DNA per test site
    b. Always prepare more DNA than needed (8-10 µL)
    c. If doing a full test plate (16 sites), prepare 128-160 µL DNA
4. Dilute test sample(s) to 50 pg/µL (or 100 pg/µL) in total volume needed using Tris-HCl
    a. Similar to calibrator DNA, need 2 µL of DNA per sample * 3 samples per test site = 6 µL DNA per test site
    b. Using 8-10 µL DNA per test site, a full plate requires 128-160 µL DNA total
5. Load primer set master mixes into wells according to map/matrix
    a. Set 96-well plate on ice, in a plate holder, with the barcode facing you
    b. Set pipette to FinalVolume-DNAVolume and use tape to prevent pipette from moving from set volume
    c. Use optical tape lid to cover wells that are not currently being filled
6. Add appropriate DNA samples to wells according to map/matrix
    a. Set pipette to DNAVolume (usually 2 µL) and use tape to prevent pipette from moving from set volume
    b. Use new tip box and load each well, using the tip that corresponds to the same position
7. Cover plate with optical tape and spin plate down at 1000 RPM for 1 min. (Larschan Lab)
    a. Remove circular centrifuge chamber and insert plate chamber
    b. After spinning plate down, check for air bubbles
    c. Remove air bubbles by spinning plate again
8. Turn on computer and qPCR machine
9. Open the qPCR computer program
    a. Start new document
    b. Accept settings
    c. Add appropriate primer sets (Reporter dye = SYBR, quencher dye = none, passive reference dye = ROX)
    d. Highlight appropriate wells and label as particular primer set
    e. Within each primer set, label wells as either standard, NTC, or "unknown' according to what they are
    f. Add dissociation stage in 'Instrument' tab
    g. Set 'Sample Volume' to correct sample volume (i.e. 15-25 µL)
10. Gently wipe bottom of qPCR plate with KimWipe, then place plate in machine with barcode facing you
11. Start qPCR cycles
12. When qPCR is complete (~2.5 hours), go to 'Analysis' tab
    a. Take notes on amplification plots and dissociation curves

b. Export results to thumb drive

13. Analyze results in Excel or R to determine whether or not each primer passes validation

Collecting salivary glands for developmental gene expression qPCR validation

Staging larvae

1. Pick and plate female larvae, and coarsely re-assign larvae to 4-5 other plates--no eye spots, early, mid, late, edge eye/drop jaw
2. Further stage larvae of interest using compound scope
    a. Place 2 slides on metal ice block, and 1 mL of Robert's buffer CR on ice
    b. Place agar plate containing coarsely assigned larvae on ice
    c. Add 2 separate drops of 40-60 µL of Robert's buffer to slide, and then place one larva on each puddle of buffer with eyespots facing up
    d. Gently place coverslip on both larvae, and place slide on ice block
    e. Repeat Steps c-d using second slide, and other larvae, totaling 4 larvae on 2 slides
    f. Remove first slide from ice block, and quickly wipe bottom of the slide
    g. Position left larva under light beam of compound scope, and record exact eyespot stage
    h. Continue to the next larva on same slide
    i. Remove coverslips, and place larvae onto appropriately labeled plates
    j. Put 2 new larvae on slide, and place coverslip on top
    k. Place slide on ice block, and process second slide
3. Place staged larvae on separate plate of given stage
4. Begin dissections immediately (within 2 hours of staging)

Salivary gland dissections for developmental gene expression qPCR– 15 pairs of salivary glands per biological replicate

1. Open laboratory entrance door to create cross-breeze when using volatile TRIzol Reagent outside of the hood
2. Place 100 µL TRIzol Reagent in microtube, and keep on ice tray
3. Aliquot 1.5 mL of Robert's buffer CR into separate microtube, and place on ice tray
4. Aliquot 250-300 µL of cold Robert's buffer CR onto slide to form a horizontally elongated puddle
5. Place 5, precisely staged female larvae, evenly spaced, across puddle
6. Starting with larva on the left, remove salivary glands from larval body
7. Clean each pair of salivary glands, removing excess tissue and fat bodies
8. Collect 5 pairs of salivary glands, and shake them
9. Repeat steps 3 through 8, placing dissected salivary glands in same TRIzol microfuge tube for a total of 15 pairs of salivary glands per biological replicate

Collecting tissue for sex-specific differential gene expression qPCR validation across *Sciara* life stages (embryos, larvae, pupae, and adults)

Notes:

- Collect at least 3 biological replicates per sex for each of *Sciara* life cycle stage
- Each biological replicate should contain ~25 mg of tissue
- Store whole samples at -80°C until enough tissue is collected to proceed to TRIzol total RNA extraction

Collecting time-laid embryos (embryo plating)

Preparing Bacto-Agar plates

1. Add 22 g Bacto-Agar (from Becton-Dickinson) to a 1500-2000 mL Erlenmeyer flask
2. Obtain 990-1000 mL of deionized millipore water in a graduated cylinder and pour ¼ - ⅓ of water into the flask
3. Swirl the flask to mix Bacto-Agar, and slowly add remaining water to clean Bacto-Agar off walls
4. Cap flask with aluminum foil, and put autoclave tape on
5. Put deionized water in autoclave tray, and place flask in autoclave tray
6. Autoclave solution on "LIQ 1L" (Liquid 1 liter)
7. Carefully remove autoclave tray with flask from autoclave using autoclave gloves, and bring solution to 55-60°C in temperature-controlled water bath
8. While waiting for temperature to cool, obtain new bag of small (50 mm x 9 mm), and medium (100 mm x 15 mm) petri dishes
9. Turn on flame to keep debris from falling down into workspace, and sterilize workspace with 70% EtOH
10. Carefully remove plates from bags, and retrieve ~55°C Bacto-Agar solution
11. Pour plates, and let agar set for at least 2 hours
12. Turn flame back on, stack plates, and pull bag down over plates
13. Close opening of bag, making it airtight, and tape closed
14. Store at 4°C

Preparing antibacterial/antifungal (anti/anti) plates

Ordering information: "HyClone Antibiotic Antimycotic Solution"

Working dose: 10 mL/L of 100X solution

Storage: 10 mL aliquots in 15 mL tubes stored at -20°C

1. Add 22 g Bacto-Agar (from Becton-Dickinson) to a 1500-2000 mL Erlenmeyer flask
2. Obtain 990-1000 mL of deionized millipore water in a graduated cylinder and pour ¼ - ⅓ of water into the flask
3. Swirl the flask to mix Bacto-Agar, and slowly add remaining water to clean Bacto-Agar off walls
4. Cap flask with aluminum foil, and put autoclave tape on
5. Put deionized water in autoclave tray, and place flask in autoclave tray

6. Autoclave solution on “LIQ 1L” (Liquid 1 liter)
7. Carefully remove autoclave tray with flask from autoclave using autoclave gloves, and bring solution to 55-60°C in temperature-controlled water bath
8. While waiting for temperature to cool, obtain new bag of small (50 mm x 9 mm), and medium (100 mm x 15 mm) petri dishes
9. Turn on flame to keep debris from falling down into workspace, and sterilize workspace with 70% EtOH
10. Carefully remove plates from bags, and retrieve ~55°C Bacto-Agar solution
11. Under flame, remove aluminum cap, and pour 10 mL of 100X anti/anti solution
12. Swirl well, but avoid creating bubbles
13. Pour plates, and let agar set for at least 2 hours
14. Turn flame back on, stack plates, and pull bag down over plates
15. Close opening of bag, making it airtight, and tape closed
16. Store at 4°C

Plating flies using CO_2 method

Notes:

- Obtain bactoagar plates, and antibacterial/antifungal plates
- Clean hood, microscope, and all working gear in hood with 70% EtOH
- Wash small paint brush, and forceps in sink with running water, followed by sterilization with 70% EtOH
- Keep sterilized forceps and brush on Kimwipe soaked in 70% EtOH
- If collecting adults, pre-weigh Eppendorf tube

1. Sterilize fly culture hood, microscope, and all equipment using Kimwipes and 70% EtOH
2. Without removing cloth stopper, put needle of CO_2 gun to mass mating vial
3. Squeeze trigger of CO_2 gun, and count to 10 to knock out flies
4. Tap bottom edge of vial on fly pad, and collect flies in one place
5. Use foot pedal to activate CO_2 release from the CO_2 pad, and keep CO_2 pad on while collecting flies onto it
6. Remove cloth stopper, and tap knocked-out flies onto CO_2 pad
7. Using paintbrush, obtain already-laid embryos from agar in vial, and transfer them to top sector of agar plate
8. Observe flies on CO_2 pad through microscope, and collect all males using forceps
9. Place collected males in pre-weighed Eppendorf tube
10. One by one, pick up female by crushing its thorax with forceps and laying it on agar ~1 inch from the top of the plate
11. Plate each of the females in neat rows and columns
12. Once all the females are plated, start a new vial

13. After all the vials are completed, move agar plate(s) under the hood. Do NOT put lid(s) on agar plate(s), as the embryos will get stuck and dried out on the lid
14. Weigh Eppendorf tube with male flies, and subtract the weight of the empty Eppendorf tube to obtain the approximate fly weight
    a) Label tube with contents, weight, date, name, etc
    b) Place tube directly in -80°C freezer

Incubation for egg laying

1. After 1 hour, check egg laying and re-squish plated flies that are moving in place or walking around
2. Allow agar plate(s) to sit for a minimum of 2 hours, and a maximum of 4 hours before transferring embryos to small anti/anti plate

Removing squished female adults

1. After allowing the eggs to lay for 2-4 hours, carefully separate the adults from the embryos under the microscope
2. Discard squished female flies in 70% EtOH soaked Kimwipe, or collect female adults in a pre-weighed Eppendorf tube
    a. If collecting female adults, obtain mass of tube with flies in it, then subtract weight of empty tube for approximate fly weight
    b. Label the tube with the contents, weight, date, name, etc., and store in -80°C freezer

Transferring embryos to small anti/anti plate

1. Label bottom of small anti/anti plate with date of collection, initials, etc.
2. Using small paintbrush, collect clumps of embryos
3. Transfer embryos to center of small anti/anti plate
4. Gently place lid on plate (no need to snap fully shut), making sure that embryos do not get stick to lid
5. Place small anti/anti plate containing embryos into empty tip box, and incubate on lab bench at room temperature for needed amount of time (2 hours, 1 day, 2 days, 3 days)
6. After the incubation period, pre-weigh an Eppendorf tube, add embryos to the tube, weight the tube again, and subtract the weight of the empty tube to obtain the embryo weight
    a. Alternatively, add embryos to tarred Eppendorf tube, and obtain embryo weight by putting tube back on scale
7. Store embryos in the -80°C freezer until TRIzol total RNA extraction

Collecting sex-specific mixed stage larvae

1. Plate larvae from mass mating jars, keeping females and males separate
2. Within each sex, separate larvae into 5 stages: pre-eyespot, early eyespot, mid-eyespot, late eyespot, EEDJ
3. Randomly sample 5 larvae from each plate and combine into labeled Eppendorf tube

    a. If there are not enough larvae to make a complete mixed stage biological replicate, then collect 5 larvae from each of the stages that are available and store at -80°C
    b. The separately larvae from each stage can be combined into a single biological replicate during the TRIzol homogenization step
4. Continue random sampling for at least 3 biological replicates of mixed stage larvae
5. Store samples at -80°C until TRIzol total RNA extraction

Collecting sex-specific mixed stage pupae

1. Plate pupae from mass mating jars, keeping females and males separate
2. Within each sex, separate pupae across the pupal stages
3. Randomly sample 25 pupae across the pupal stages and combine into labeled Eppendorf tube
4. Continue random sampling for at least 3 biological replicates of mixed stage pupae
5. Store samples at -80°C until TRIzol total RNA extraction

Collecting sex-specific mixed stage adults

1. Use CO_2 pad to knock out adults
2. Randomly sample 50 male adults and combine into labeled Eppendorf tube
3. Randomly sample 25 female adults and combine into labeled Eppendorf tube
4. Store samples at -80°C until TRIzol total RNA extraction

TRIzol total RNA extraction for gene expression qPCR

Caution: TRIzol reagent should be worked with under the fume hood while wearing lab coat, gloves, and eye protection. See TRIzol manual (https://tools.thermofisher.com/content/sfs/manuals/trizol_reagent.pdf)

1. Collect ~25 mg sample (embryos, larvae, pupae, adults) into one RNase-free Eppendorf tube
2. Homogenize sample in 250 µL TRIzol reagent (under fume hood) using sterile blue pestle
3. When tissue is homogenized thoroughly, add 250 µL TRIzol more (total volume of 500 µL) and do second/final homogenization
4. Snap lid closed, and let homogenized material sit for 5 min.
    a. Material can safely sit for several hours at RT
5. Centrifuge sample at 12,000xg for 10 min. to remove bulk fats, polysaccharides, and extracellular material
6. Remove and discard top fatty layer
7. Transfer cleared supernatant to a new RNase-free Eppendorf tube leaving pellet behind
8. Add 100 µL of chloroform (200 µL chloroform per 1 mL TRIzol) to sample and cap lid securely

9. Shake tube vigorously by hand for 20 seconds
10. Let sample sit at RT for 5 min.
11. Centrifuge sample at 12,000xg for 15 min.
12. Remove top aqueous phase, which contains RNA, and transfer into another RNase-free Eppendorf tube
    a. Do not include any of the interphase or organic phase
13. Wipe surface with RNase Zap and change gloves
14. Add 250 µL of 100% isopropanol (500 µL of 100% isopropanol per 1 mL TRIzol)
15. Incubate at RT for 30 min.
16. Centrifuge at maximum speed for 15 min.
17. Remove supernatant, leaving RNA pellet
18. Wash the pellet with 500 µL 75% EtOH (1 mL 75% EtOH per 1 mL TRIzol)
    a. RNA can be stored in 75% EtOH at -20°C for at least 1 year
19. Set heat block to 55-60°C for re-suspension step
20. Briefly vortex sample and centrifuge at 7500xg for 5 min.
21. Discard supernatant and repeat 75% EtOH wash
22. Air dry pellet for 5-10 min.
    a. Do not allow RNA to dry completely
23. Re-suspend RNA pellet in 20-100 µL UPW by passing solution over pellet several times using pipette tip
24. Incubate sample on heat block set to 55-60°C for 10-15 min.
25. Transfer sample to ice and proceed to next part of workflow
    a. Alternatively, store RNA sample at -20°C or -80°C

Quantify Total RNA (Nanodrop)

Notes:

- 260/280 ratio of ~2.0 is generally considered pure RNA, but this value can vary ~0.2-0.3 in either direction just from differences in pH
- 260/230 should minimally be ~2.0-2.2—if lower than ~2.0, be suspicious of contaminants (i.e. EDTA, carbohydrates, and/or phenol)
- Nanodrop is a good first approximation to see how much to dilute the sample for a Qubit quantification—the top of the Qubit range is 100 ng/µL, which will require a 200-fold dilution in the Qubit step; if the Nanodrop trace says there is >100 ng/µL, the sample must be diluted

1. First, clean Nanodrop with dH_2O and KimWipe
2. Initialize the Nanodrop with dH_2O and clean with Kimwipe
3. Set settings to RNA-40
4. Use 2 µL UPW as 'Blank' (RNA is in UPW)
5. Clean Nanodrop with KimWipe
6. Use 2 µL RNA sample to measure RNA

Quantify Total RNA (Qubit)

Notes:

- High confidence detection range of the amount of RNA after dilution is 25 pg/µL to 500 pg/µL

1. Set up the number of 0.5 mL Qubit tubes needed for standards and sample(s)
    a. numTubes = numSamples + 2 standards
2. Label the lids of each tube
3. Make Qubit working solution (WS) by diluting Qubit RNA reagent 1:200 in Qubit RNA buffer
    a. Prepare enough WS to accommodate all standards and samples
    b. Each standard requires 190 µL of WS, while each sample requires 199 µL of WS
    c. Approximate ~200 µL * numTubes
4. Load 190 µL WS into both standard tubes
5. Add 10 µL of the appropriate standard to its correspondingly labeled tube
    a. Mix by vortexing for 2-3 seconds, being careful not to create bubbles
6. Load 199 µL of WS into each sample tube, then add 1 µL of each sample to its correspondingly labeled tube
7. Allow tubes to incubate at RT for 2 min.
8. On the home screen of the Qubit Fluorometer, press 'RNA,' then select 'RNA' again as the assay type
9. Run a new calibration by inserting Standard #1 tube into the machine
10. Close the lid, then select 'Read'
11. Remove Standard #1 and insert Standard #2
12. Again, close the lid, press 'Read,' then remove Standard #2
13. Insert sample tube into the Qubit, close the lid, and press 'Read'
    a. Upon completion of the measurement, remove Qubit tube immediately
14. The result, "QF," will be displayed on the screen
    a. The QF value corresponds to the concentration after the dilution
    b. [Sample] = QF * (200/x), where x is the volume of sample in µL used in the dilution
15. Insert the next sample, and press 'Read next sample.' Measure, remove, then record value
16. Repeat sample readings until all samples have been read

Preparing 2% agarose gels to confirm RNA quality through gel electrophoresis

1. Set up gel molds with appropriate combs
2. Obtain 1g agarose gel in 250 mL Erlenmeyer flask
3. Add 50 mL 1X TAE to Erlenmeyer flask and gently swirl
4. Microwave solution, carefully swirling flask after bubbles form
5. Run Erlenmeyer flask under cold water to cool flask

6. Pour gels and let stand for at least 30 min.
7. Load 10 µl 1 kb dsDNA ladder in first lane
8. Load 500 ng RNA sample with 6X loading dye in second lane
9. Run gel for 1 hour at 90V
10. Stain in EtBr solution for 15 min., then de-stain for 15 min.
11. Visualize gel

DNA-free DNase treatment (from Ambion by Life Technologies)

1. Prior to beginning DNA-free DNase treatment, autoclave 0.5 mL tubes and thaw reagents on ice (stored at -20°C)
2. Set ThermoMixer to 37°C
3. Dilute RNA to 10 µg in 50 µL reaction
4. Add 0.1 volume 10X DNase I Buffer and 1 µL rDNase I to RNA, and mix gently
5. Incubate at 37°C for 15 min.
6. Add 2 µL resuspended DNase Inactivation Reagent and mix well
    a. Resuspend DNase Inactivation Reagent by flicking or vortexing
7. Incubate for 2 min. at RT, mixing at least 2-3 times during incubation to redisperse DNase Inactivation Reagent
8. Centrifuge at 10,000xg for 1.5 min. and transfer RNA to fresh tube
    a. Carefully transfer supernatant and airing on the side of leaving some supernatant behind
9. Re-centrifuge supernatant at 10,000xg to ensure removal of DNase Inactivation Reagent
10. Carefully transfer supernatant and proceed workflow or store at -20°C

cDNA synthesis from DNA-free RNA

1. Set PCR machine to 65°C
2. Prepare first mix in PCR tube:
    11 µL RNA (200-500 ng)
    1 µL random hexamer primers
    1 µL 10 µM dNTP solution
3. Incubate first mix at 65°C for 5 min., then on ice for 2 min.
4. Prepare second mix while first mix is incubating:
    4 µL 5X FSS reaction buffer
    1 µL 0.1 DTT
    1 µL murine RNase inhibitor
    1 µL SSIII RT
5. Spin first mix tube and place on ice
6. Combine all first and second mix in PCR tube
7. Run PCR program
    25 °C, 10 min.
    50 °C, 50 min.
    85°C, 5 min.
    4°C, forever

Quantify cDNA (Qubit)

1. Set up the number of 0.5 mL Qubit tubes needed for standards and sample(s)
    - numTubes = numSamples + 2 standards
2. Label the lids of each tube
3. Make Qubit working solution (WS) by diluting Qubit ssDNA dye 1:200 in Qubit ssDNA buffer
    - Prepare enough WS to accommodate all standards and samples
    - Each standard requires 190 µL of WS, while each sample requires 199 µL of WS
    - Approximate ~200 µL * numTubes
4. Load 190 µL WS into both standard tubes
5. Add 10 µL of the appropriate standard to its correspondingly labeled tube
    - Mix by vortexing for 2-3 seconds, being careful not to create bubbles
6. Load 199 µL of WS into each sample tube, then add 1 µL of each sample to its correspondingly labeled tube
7. Allow tubes to incubate at RT for 2 min.
8. On the home screen of the Qubit Fluorometer, press 'RNA,' then select 'RNA' again as the assay type
9. Run a new calibration by inserting Standard #1 tube into the machine
10. Close the lid, then select 'Read'
11. Remove Standard #1 and insert Standard #2
12. Again, close the lid, press 'Read,' then remove Standard #2
13. Insert sample tube into the Qubit, close the lid, and press 'Read'
    - Upon completion of the measurement, remove Qubit tube immediately
14. The result, "QF," will be displayed on the screen
    - The QF value corresponds to the concentration after the dilution
    - [Sample] = QF * (200/x), where x is the volume of sample in µL used in the dilution
15. Insert the next sample, and press 'Read next sample.' Measure, remove, then record value
16. Repeat sample readings until all samples have been read

<u>Reference gene primer validation for differential gene expression qPCR experiments</u>
<u>(10 µl recipe)</u>

Notes:

- Check for starting materials at beginning of day (autoclaved Eppendorf tubes, female adult DNA, etc.)
- Sign up for qPCR machine for time expected to be done preparing qPCR plate

1. Make 1 µM primer solutions (-20°C freezer)
    a. Add 180 µL of UPW to 12 pre-labeled Eppendorf tubes

b. Add 20 µL of corresponding 10 µM primer to each tube
c. Thaw primers on ice
d. Set up workbench (fill 3 ice buckets, empty waste container, wipe down surfaces with EtOH, etc.)

2. For gDNA dilutions, working concentration is 1 ng/µL and 2 ng will be used per reaction
    a. First, Qubit cDNA to obtain concentration
    b. Dilute cDNA to 1 ng/µL in sufficient volume for all reactions (24 reactions/plate) and dilution series to obtain 1:1 cDNA
        i. Need at least 48 µL at 2 µL per reaction; taking into consideration 24 reactions per plate, 25 µL for dilution, and pipetting error, make 100 µL at 1 ng/µL
    c. Make 1:4 by combining 25 µL 1:1 with 75 µL UPW
    d. Make 1:16 by combining 25 µL 1:4 with 75 µL UPW
    e. Make 1:64 by combining 25 µL 1:16 with 75 µL UPW
3. Prepare master mixes on ice— 8 reactions per primer pair; up to 12 primer pairs per plate; make enough master mix for 10 reactions
    a. Label 12 Eppendorf tubes with primer pair names
    b. Tape pipette to appropriate volume and follow the recipe below:

        9 reactions using 1 µM:
        9 µL UPW (add first)
        45 µL 2X SYBR Green
        9 µL of 1 µM forward primer
        9 µL of 1 µM reverse primer
        Total volume of 72 µL

    c. After adding final component of master mix, pipette up and down to mix
    d. Shortly spin master mixes down
    e. Place 1 µM primer stock solutions back in -20°C freezer
4. Load columns of plate with corresponding primer master mixes
    a. Place plate on ice in empty tip platform with barcode facing you
    b. Add primers left to right, one column at a time
    c. Add 8 µL of master mix in designated well according to well map
    d. Keep all plate columns to the right of current column covered with optical tape without exposing sticky part
    e. Only pipette over covered regions and current well
    f. Drop master mix solution along side of well
5. Load rows with corresponding DNA concentrations (high to low)
    a. Add cDNA top to bottom, one row at a time
        i. Rows A and B = 1:1 (1:1)
        ii. Rows C and D = 1:10 (1:4)
        iii. Rows E and F = 1:100 (1:16)
        iv. Rows G and H = 1:1000 (1:64)
    b. Add 2 µL of cDNA to each well, dropping solution along side of well
6. Seal plate using sticky side of the optical tape
7. Spin plate down at 1000 RPM for 1 min. (Larschan Lab)

    a. Remove circular centrifuge chamber and insert plate chamber
    b. After spinning plate down, check for air bubbles
    c. Remove air bubbles by spinning plate again
8. Turn on computer and qPCR machine
9. Open the qPCR computer program
    a. Start new document
    b. Accept settings
    c. Add appropriate primer sets (Reporter dye = SYBR, quencher dye = none, passive reference dye = ROX)
    d. Highlight appropriate wells and label as particular primer set
    e. Within each primer set, label wells as either standard, NTC, or "unknown' according to what they are
    f. Add dissociation stage in 'Instrument' tab
    g. Set 'Sample Volume' to correct sample volume (i.e. 10-25 µL)
10. Gently wipe bottom of qPCR plate with KimWipe, then place plate in machine with barcode facing you
11. Start qPCR cycles
12. When qPCR is complete (~2.5 hours), go to 'Analysis' tab
    a. Take notes on amplification plots and dissociation curves
    b. Export results to thumb drive
13. Analyze results in Excel or R to determine whether or not each primer passes validation