Ecdysone-Regulated DNA Amplification in *Sciara coprophila*

By

Léo Kadota

B. Sc., Brown University, 2016

Thesis

Submitted in partial fulfillment of the requirements for the
Degree of Master of Science in the Department of Molecular Biology, Cell Biology, & Biochemistry at Brown University

PROVIDENCE, RHODE ISLAND

MAY 2017

This thesis by Léo Kadota is accepted in its present form
by the Department of Molecular Biology, Cell Biology, & Biochemistry as
satisfying the thesis requirements for the degree of Master of Science

Date_____ _____

Susan A. Gerbi, Ph.D., Advisor

Date_____ _____

Stephen L. Helfand, M.D., Second Reader

Approved by the Graduate Council

Date_____ _____

Andrew. G. Campbell
Dean of the Graduate School

AUTHORIZATION TO LEND AND REPRODUCE THE THESIS

As the sole author of this thesis, I authorize Brown University to lend it to other institutions or individuals for the purpose of scholarly research.

Date_____ _____

Léo Kadota, Author

I further authorize Brown University to reproduce this thesis by photocopying or other means, in total or in part, at the request of other institutions or individuals for the purpose of scholarly research.

Date_____ _____

Léo Kadota, Author

Acknowledgements

The first biology course I have ever taken was BIOL 0200 at Brown University. Everything that I have learned about biology, I have learned through my professors and peers at this institution. Accordingly, beyond acknowledging those who have contributed directly to my ability to complete this thesis, this page seems like an appropriate place to acknowledge even those who first taught me four years ago what a protein is. The work in this thesis owes credit to several key members of the Gerbi Laboratory, evidently Professor Gerbi herself, and John Urban, Jacob Bliss, and Yutaka Yamamoto, the major repertoires of knowledge who I turned to throughout my time in the lab. My thanks also extends to the other lab members – Ben Doughty, Audrey Lee, Julia Leung, Miiko Sokka – for being around to regularly bounce ideas back and forth, Judith Bender and Elaine Butler for coordinating the MCB Master's program this past year, and importantly, my parents Camille Bureau and Satoru Kadota for supporting my studies abroad from Canada over the past five years.

Table of Contents

Section	***Page***
Abstract	1-2
Introduction	3-17
Ecdysone-Induced DNA Amplification	18-37
Finding a Small ncRNA Complementary to the 1kb II/9A ORI	38-43
Discussion	44-47
Supplementary Tables	48-49
Methods	50-56
References	57-61

List of Figures and Tables

Figure #, Description		***Page***
1	DNA replication initiation events	4
2	DNA amplification onionskin and puff	8
3	Map of *Sciara* DNA puff II/9A locus	9
4	Normal developmental progression of *Sciara*	10
5	Developmental changes in DNA amplification and transcription at the II/9A locus	12
6	Ecdysone-induced DNA amplification at II/9A locus, 24 hrs post-injection (Foulk et al. 2006)	15
7	Ecdysone-induced DNA amplification at II/9A, 24 hrs post-injection (thesis validation)	20
8	Ecdysone-induced whole-body phenotypic changes	22
9	Ecdysone-induced eyespot phenotypic changes	24
10	Ecdysone-induced DNA amplification at all candidate DNA puffs, 24 hrs post-injection	28
11	Ecdysone-induced DNA amplification at all candidate DNA puffs, 24 hrs post injection + DNA amplification in normal Edge-Eye larvae	31
12	Ecdysone-induced DNA amplification at all candidate DNA puffs, 48 hrs post-injection	32
13	Ecdysone-induced DNA amplification at all candidate DNA puffs, 16 hrs post-injection	34
14	Ecdysone-induced phenotypic changes, 70-120 hrs post-injection	36
15	Optimization of small RNA extraction and enrichment	40
Table #, Description		
1	Chromosomal positions of candidate DNA amplicons	27
1	(Supplementary) Ecdysone-induced fold-enrichment and standard deviation for all loci and time points	48
2	(Supplementary) P-values for Supplementary Table 1	49

Abstract of 'Ecdysone-Regulated DNA Amplification in *Sciara coprophila'*, by Léo Kadota, Sc.M., Brown University, May 2017.

The eukaryotic genome contains thousands of origins (ORIs) of DNA replication, which are sequences where DNA synthesis begins in S phase of the cell cycle. The initiation of DNA replication begins in G1 phase in a tightly regulated process that is coordinated with cell cycle progression. This ensures that an ORI fires no more than once per cell cycle in order to evenly duplicate the genome that is subsequently evenly divided to daughter cells during mitosis. De-regulation of DNA replication initiation can lead to DNA re-replication, where an origin fires more than once during S phase, producing more than the diploid copy number. Locus-specific re-replication, also called DNA amplification, can lead to genomic instability, a hallmark of certain human cancers. DNA amplification of tumorigenic genes has also been documented in the cells of breast cancer tumors. It is therefore of general biological and medical importance to understand the mechanisms leading to DNA amplification.

It is hard to study the initiating events of DNA amplification in cancer cells. However, DNA amplification occurs as part of normal development during 4th larval instar in the larval salivary gland chromosomes of the fungus gnat *Sciara coprophila.* In human diseases, DNA amplification is a rare event and is therefore difficult to study experimentally. *Sciara* is a model organism that provides a unique opportunity to elucidate the mechanisms of DNA amplification *in vivo*. In *Sciara*, DNA amplification occurs at eighteen amplicon loci distributed across its four chromosomes. There is accumulating evidence that the steroid hormone ecdysone,

the master regulator of insect development, acts through the ecdysone receptor as the amplification trigger for DNA amplification. This thesis describes experiments in which ecdysone injection into pre-amplification stage *Sciara* larvae prematurely induced DNA amplification, as measured by quantitative PCR, at all DNA amplicon loci. These observations support the hypothesis that ecdysone and its receptor act as the amplification trigger in *Sciara*. This thesis also details ongoing experiments aimed at determining whether ecdysone induces the transcription of small non-coding RNAs complementary to the amplicon origin, which could recruit replisome machinery to the amplicon ORIs to promote DNA amplification.

1. Introduction

The replication of deoxyribonucleic acid (DNA) in eukaryotic cells initiates from one of the thousands of replication origins (ORIs) distributed throughout the genome (Mesner et al. 2011, Miotto et al. 2016). Normally, several regulatory mechanisms are in place that ensure only a single activation of an ORI per cell cycle (Dutta & Bell 1997, Bielinsky & Gerbi 2001, Bell 2002, Aladjem & Fanning 2004, DePamphilis et al. 2006, Aladjem 2007, DePamphilis & Bell 2010). The resulting duplicated genome is then equally divided among two daughter cells during mitosis, ensuring constancy of DNA copy number throughout cellular divisions The activation, or the 'firing', of an ORI requires recruitment of DNA polymerase once per synthesis (S) phase. Prior to loading of the DNA polymerase, the pre-replication complex (pre-RC) is assembled at the ORI during the G1 phase of the cell cycle when cyclin dependent kinase (CDK) levels are low (Figure 1). The formation of the pre-RC begins with the loading of the six-polypeptide Origin Recognition Complex (ORC), which in turn acts as a landing pad for cell division cycle 6 protein (Cdc6). Next, two hexameric helicases (minichromosome maintenance 2-7)(Mcm2-7)), in their inactive form, are sequentially loaded along with Cdt1 (Yardimci & Walter 2014). The origin is subsequently considered 'licensed', meaning that it is competent for initiation of replication (Gerbi et al. 2002).

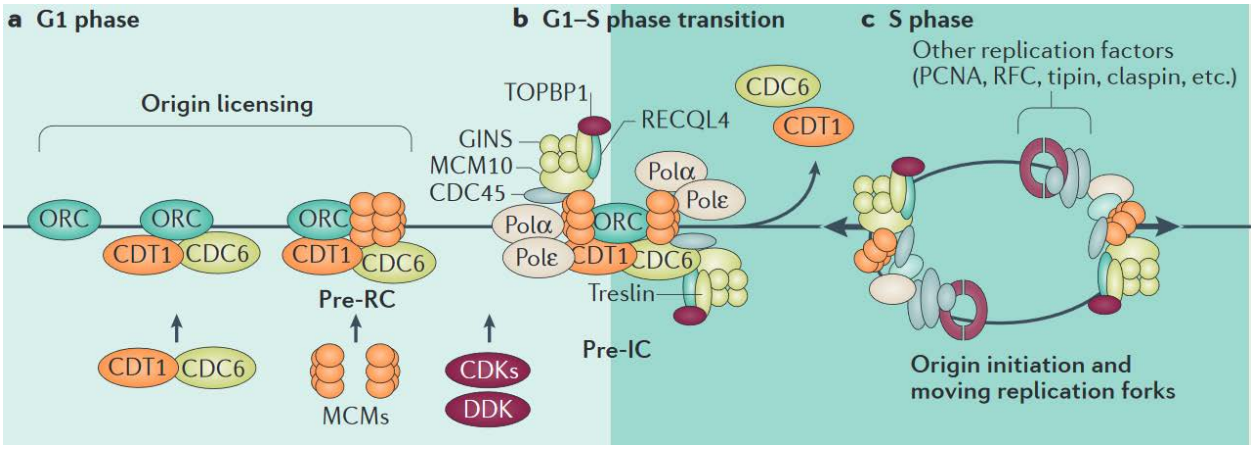

Figure 1 – DNA replication initiation events (Figure from Aladjem & Redon 2016). In G1 phase, the pre-replication complex (pre-RC) is formed when CDK levels are low. An increase in CDK levels during transition into S phase converts the pre-RC into the initiation complex (IC). The coupling of replication initiation events with the progression of the cell cycle ensures than an origin fires no more than once per cell cycle.

When CDK levels rise at the end of the G1 phase, Cdc6 and Cdt1 then dissociate from the pre-RC and Cdt1 is sequestered by geminin (Wohlschlegel et al. 2000). The ORI is left with only ORC and the double Mcm2-7 hexamers, and then joined by the remaining components of the replisome, including Cdc45 and the GINS complex, in a process dependent on CDK-mediated phosphorylation (Heller et al. 2011, Aladjem & Redon 2016). This completes the conversion of the pre-RC into the initiation complex (IC), activating the helicases. This activation allows the ORI to 'fire' during S phase to initiate DNA synthesis, and the two helicases migrate bidirectionally outward. The resulting unwinding of the DNA at the ORI allows DNA polymerase access to bind to the chromatin and catalyze DNA synthesis. Bidirectional replication continues until adjacent replication forks

converge. CDK levels remain high during S phase and G2 phase, preventing pre-RCs from forming a new IC due to inhibition of Cdt1-Orc6 interactions by CDK, which in turn prevents Mcm2-7 loading (Chen & Bell 2011). Inhibition of IC formation and ORI licensing by CDK during G1, followed by CDK-dependent origin activation in S phase, provides a strict regulatory replication mechanism coupled with cell-cycle signaling that prevents more than one genome duplication per cell cycle (Bielinsky & Gerbi 2001, Aladjem & Redon 2016).

Deregulation of these DNA replication controls leads to aberrant ORI firings, causing chromosomal breaks and fusions. Such genomic instability is a common hallmark of certain human cancers, suggesting that deregulating origin firing can promote cancer progression (Blow & Gillespie 2008, Alexander JL et al. 2015) In yeast, removing global controls against re-replication results in genome-wide re-firing of origins (Gopalakrishnan et al. 2001, Nguyen et al. 2001, Brewer et al. 2001, Diffley 2010, Green et al. 2010, Kiang et al. 2010). Aberrant ORI firings have also been observed in higher eukaryotes when Cdt1 is upregulated or its inhibitor geminin is depleted (Li & Bloe 2005, Saxena & Duatta 2005, DePamphilis et al. 2006). In certain human cancers, proteins involved in ORI licensing such as Mcm2-7and Cdt1 are inappropriately expressed, suggesting that loss of replication initiation controls promotes tumorigenesis (Blow & Gillespie 2008, Hanahan & Weinberg 2011). When the entire genome duplicates without mitosis, this results in an increased ploidy number in the cell, such as in the polytene chromosomes of insect salivary glands that arise from this endoduplication process. In addition, in

certain instances, locus-specific DNA re-replication results in DNA amplification where that locus has more than the normal copy number of DNA relative to the rest of the genome. DNA amplification of oncogenes have been observed in amplicome analyses of breast tumors, underscoring the importance of understanding its mechanism (Nikolsky et al. 2008). It is currently unknown how certain ORIs, within the subset of the thousands of ORIs that exist in the eukaryotic genome, are exclusively targeted and replicated for DNA amplification.

Only two cases of DNA amplification that occur as part of normal developmental are currently known, both in Dipteran flies: the DNA puffs of the late larval salivary gland polytene chromosomes of Sciarid flies and the '*Drosophila* amplicon in follicle cells' (DAFCs) found at the chorion loci and a few other loci (Tower 2004, Claycomb & Orr-Weaver 2005, Nordman & Orr-Weaver 2012). These two systems thus serve as excellent *in vivo* model systems to study the molecular factors that regulate DNA replication initiation. In *Drosophila*, there are six DAFCs that undergo gene amplification during late oogenesis (Kim et al. 2011, Kim & Orr-Weaver 2011). The developmental timing of origin activation and elongation of replication forks vary by DAFC (Kim & Orr-Weaver 2011). DNA amplification in DAFCs utilize the same replication machinery and CDK regulation as non-amplifying eukaryotic cells (Tower 2004). During the 4th larval instar of *Sciara coprophila*, the salivary gland chromosomes initially undergo 11 and 12 rounds of endoduplication in males and females respectively, producing copy

numbers of 4096C in males and 8192C in females. There is no intervening mitosis between each round of endoduplication, and all daughter chromatids remain synapsed together to form giant polytene chromosomes (Rasch 1970b). Superimposed on the last round of endo-reduplication, DNA amplification occurs at eighteen DNA puff loci (Poulson & Metz 1938, Crouse & Keyl 1968, Gabrusewycz-Garcia 1964, Rasch 1970a, 1970b, 1971) distributed across the four chromosomes (three autosomes + one sex chromosome). DNA amplification produces an 'onionskin structure' of nested replication forks which are prone to breakage, suggesting that DNA re-replication events may be one mechanism that drives cancer-promoting genomic instability (Figure 2). The DNA amplicon loci in *Sciara* undergo chromatin decondensation and intense transcription at the locus resulting in 'DNA puffs' after the occurrence of DNA amplification, as studied in DNA puff II/9A, the largest and earliest appearing DNA puff (Wu et al. 1993) (Figure 2). Although all DNA amplicons in *Sciara coprophila* form puffs, not all puffs are DNA puffs. There are also RNA puffs distributed throughout the chromosomes which do not show amplified DNA loci relative to the rest of the genome. Chromatin puffs are also observed in *Drosophila*, but these are exclusively RNA puffs.

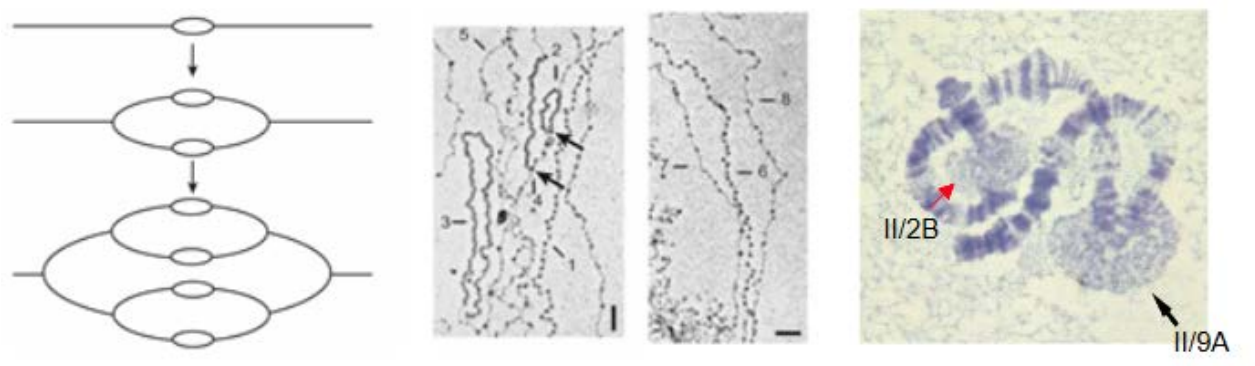

Figure 2 – (Left) 'Onionskin' DNA structure of nested replication forks resulting from repeated origin firings during a single S phase. (Center) Electron micrographs of Miller spreads of amplification-stage Sciara larval salivary gland chromatin. Arrows point to replication forks in the onionskin structure. (Right) Morphological puffing of the two largest Sciara DNA puffs, II/2B and II/9A, associated with high transcriptional activity

Endoduplication followed by locus-specific DNA amplification serves important developmental functions in *Drosophila* and *Sciara*. It allows for the production of massive amounts of DNA template to facilitate large-scale synthesis of proteins important for development. In *Drosophila* ovarian follicle cells, genes that encode structural proteins and cross-linking enzymes in the chorion are positioned at the peaks of amplicons on the third and X chromosome (Spradling 1981). Ablating gene amplification in *Drosophila* by mutating replication factors such as ORC2, MCM6, and DBF4 results in the laying of inviable eggs with thin chorions (Landis et al. 1997, Landis & Tower 1999, Schwed et al. 2002, Kim and Orr Weaver 2011). The decrease in DNA template for transcription of chorion

genes is thought to be the cause of the female sterility, underscoring the developmental importance of DNA amplification. In *Sciara*, past studies have mostly focused on the largest and earliest-appearing DNA puff, puff II/9A (chromosome II, position 9A) (Gabrusewycz-Garcia 1964, Rasch 1970a). The DNA sequence of II/9A reveals two transcription units, II/9-1 and II/9-2, that share 85% homology and encode putative alpha-helical coiled coil proteins that are thought to be secreted and contribute to the formation of the pupal cocoon (Figure 3) (DiBartolomeis & Gerbi 1989).

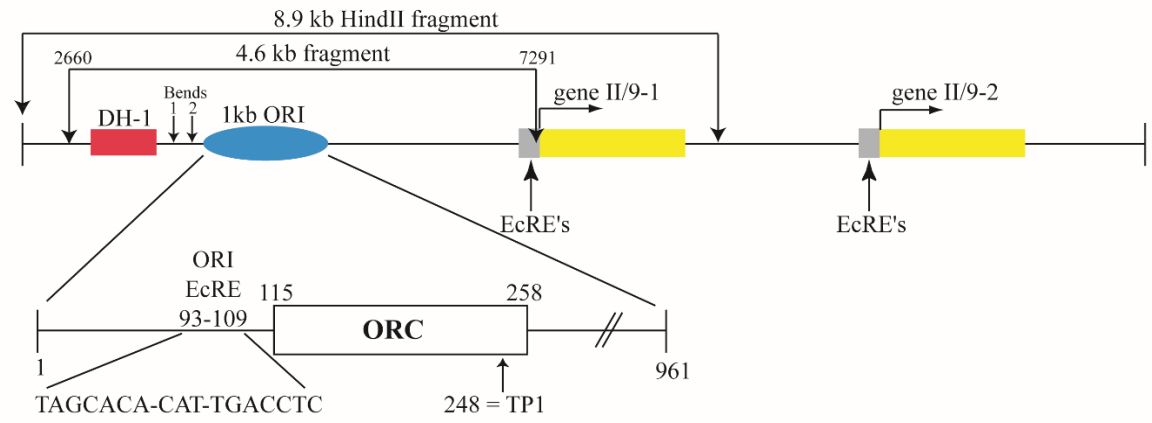

Figure 3 – Map of the Sciara DNA puff II/9A locus. Re-replication events during amplification stages initiates in the 1kb ORI, which contains and ORC binding site directly downstream of a putative ecdysone response element (EcRE), the binding site for the ecdysone receptor. Genes II/9-1 and II/9-2 encode predicted pupal cocoon proteins.

In *Sciara*, DNA amplification starts in late 4th larval instar at the 'Pre-Eyespot' stage. *Sciara* larvae are staged according to the number and shape of their

eyespots. The staging convention is: (# eyespots along longest row) x (# rows - 1). The progression of larval stages is typically characterized as: Pre-Eyespot → Early Eyespot → 8x4 → 10x5 → 12x6 → 14x7 → Edge-Eye → Drop-Jaw (Figure 4). For example, a 12x6 stage larva has twelve eyespots along the bottom row, and has seven rows of eyespots. At the Edge-Eye stage, larval eyespots coalesce and migrate laterally. At the Drop-Jaw stage, the jaw is literally shed from the larva along with the cuticle as the larva transitions into a pupa.

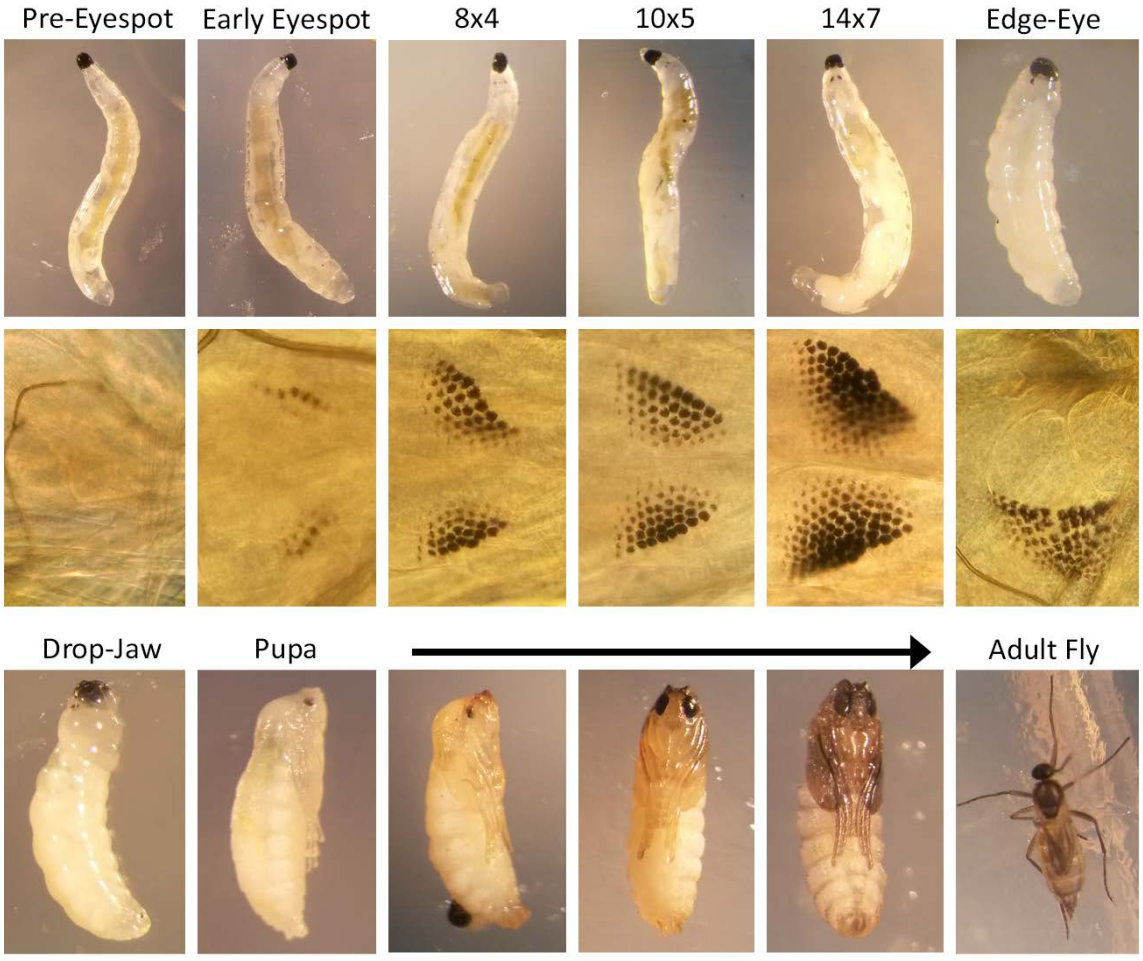

Figure 4 – Normal developmental progression of Sciara. The larval stages ranges between Pre-Eyespot and Drop-Jaw. The number and shape of the eyespots are

used to stage the larvae, and correlate with specific levels of DNA amplification. The transition between 14x7 and Edge-Eye involves the coalescing and lateral migration of eyespots. During the Drop-Jaw stage, the jaw is literally shed from the body along with the cuticle during the transition to the pupa.

At the II/9A puff, DNA amplification begins at the Early-Eyespot stage and continues to increase steadily through 14x7 stage, reaching approximately 17-fold amplification, corresponding to four additional rounds of replication. (Gerbi et al. 1993, Wu et al. 1993, Foulk et al. 2006). Given that the female polytene chromosome is endoduplicated to 8192C prior to amplification, this means that the II/9A locus reaches a copy number of nearly 140 000 C. At the 14x7 stage, there is a rapid burst of transcription of genes encoded within II/9A, which include the putative pupal cocoon proteins II/9-1 and II/9-2 (DiBartolomeis & Gerbi 1989, Gerbi et al. 1993). This burst of transcription immediately prior to the larva-pupa transition is consistent with the hypothesis that DNA amplification promotes increased synthesis of pupal cocoon proteins. However, it is not known whether genes in all eighteen DNA puffs contribute to pupation in *Sciara*, though this can be deduced from studies in the related species *Rhynchosciara*. In *Drosophila,* only two of the six DAFCs contain chorion genes, and although all six DAFCS map to regions containing gene, not all of these genes are highly expressed and most highly expressed genes are not amplified (Kim et al. 2011).

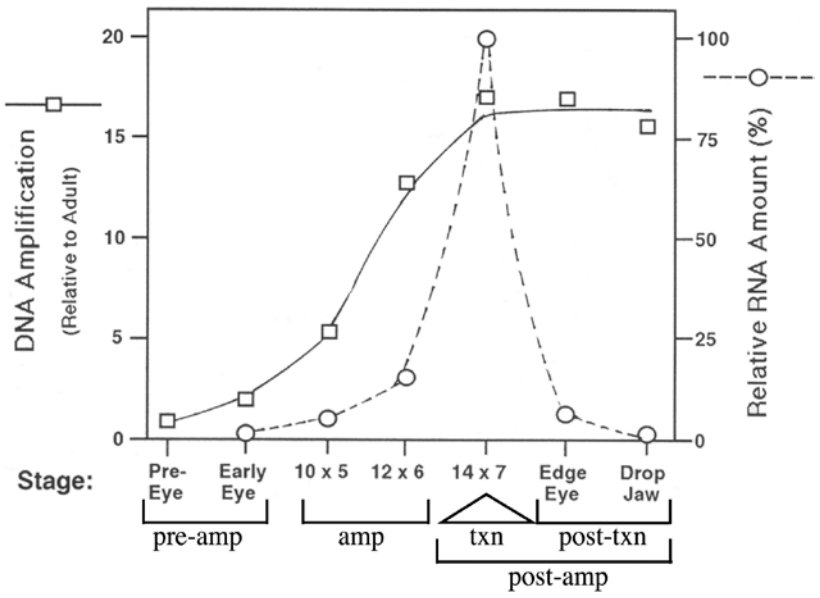

Figure 5 – Changes in DNA and RNA content at the II/9A locus throughout larval development (Gerbi et al. 1993). DNA amplification precedes increase in transcription. Transcription spike at the 14x7 stage. In this figure DNA amplification levels, determined by quantitative Southern blot, plateaus at ~17 fold after the 14x7 stage, but more recent data presented in this thesis suggests that DNA amplification levels continue to increase to ~32 fold in the Edge-Eye stage.

The molecular mechanisms responsible for initiating repeated origin firing at the DNA amplicon loci are not well understood. Somehow, the origins of the six *Drosophila* DAFCs and eighteen *Sciara* DNA puffs are selectively targeted by ORC for DNA amplification out of the thousands of origins distributed throughout the genome. An essential component of understanding ORC selectivity to the amplicons is mapping the origins of DNA amplification at the nucleotide level. In this respect *Drosophila* research falls short compared to *Sciara*. The origin of

DAFC-66, the largest *Drosophila* follicle cell amplicon on the third chromosome, has only been narrowed down at low resolution to a region spanning several thousand bases (Delidakis & Kafaros 1989, Heck & Spradling 1990, Claycomb et al. 2002, Remus et al. 2004, Kim & Orr-Weaver 2011). In *Sciara*, the II/9A amplification start site for leading strand synthesis has been mapped to the single nucleotide level, and ORC binds adjacent to this site within a 1kb ORI (Bienz-Tadmor et al. 1991, Liang et al. 1993, Liang et al. 1994). Additionally, this start site is adjacent to an ecdysone-response element (EcRE) that is capable of binding the *Sciara* ecdysone receptor (ScEcR), suggesting the steroid hormone ecdysone, also known as the 'master regulator of development', and the ecdysone receptor are involved in regulating DNA amplification (Foulk et al. 2006). The receptor for ecdysone is an obligate heterodimer of ecdysone receptor and ultraspiracle (USP). Indeed, accumulating evidence points towards the ecdysone receptor as the *Sciara* amplification trigger (Foulk et al. 2006, Foulk et al. 2013, Liew et al. 2013). Immunofluorescence experiments using antibodies against ScEcR-A, isoform A of ScEcR, showed that ScEcR-A localizes to DNA puff loci at the start of amplification. Consistent with its role as a transcription factor, ScEcR-A also localizes to RNA puffs and other genomic loci (Liew et al. 2013).

Additional evidence that ScEcR may be the amplification factor comes from a series of ecdysone-injection experiments. H^3-thymidine labelling of DNA synthesis in salivary glands 24 hours post-ecdysone injection in pre-amplification stage larvae has shown that exogenous ecdysone can prematurely induce both

the morphological puffing and DNA synthesis at DNA puff loci (Crouse 1968). However, a quantitative analysis was not performed and whether DNA amplification was prematurely induced was not determined in that study. Ecdysone injection in pre-amplification stage larvae also induces premature ScEcR-A binding to DNA puff loci and premature ~16 fold amplification of the II/9A locus as measured by qPCR (Foulk et al. 2006) (Figure 6). This ecdysone-injection qPCR experiment was only done for II/9A because only the II/9A DNA puff sequence was available at the time for primer design. In order to provide further support that ecdysone and its receptor are mechanistically involved in DNA amplification, there is still a need to repeat this experiment for the remaining DNA puffs. This is accomplished in the work described in this thesis, thanks to the recently-completed *de novo* genome assembly of *Sciara* by PhD student Dr. John Urban. The assembled genome identified fourteen candidate DNA puff sequences for which qPCR primers were designed, allowing for ecdysone-injection experiments to be done to quantify ecdysone-responsiveness of all puffs.

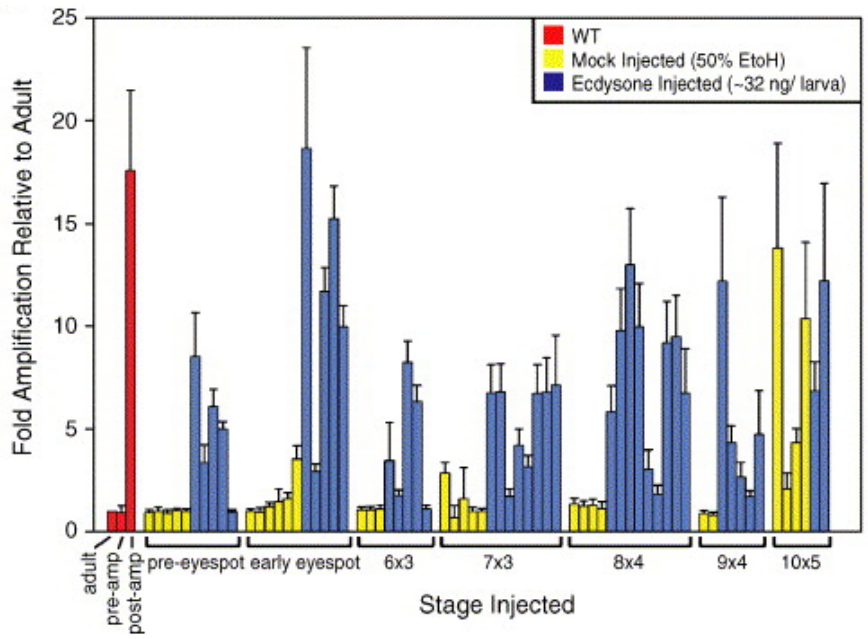

Figure 6 – qPCR results from Foulk et al. 2006 showing that ecdysone prematurely induces DNA amplification in pre-amplification stage larvae at the II/9A DNA puff locus. Other DNA puff loci were not investigated due to unavailability of the genome sequence for qPCR primer design. This thesis details qPCR experiments extending these experiments to all DNA puff loci for three time points, made possible by recent completion of the Sciara genome.

How might ecdysone drive DNA amplification at the DNA puff loci? Levels of ecdysone produced by the prothoracic glands in Sciarids are dependent on developmental stage, which may influence the onset of amplification (Candido-Silva et al. 2015). One mechanistic hypothesis is that the EcR/USP receptor complex is bound by ecdysone, inducing its localization to EcREs adjacent to DNA puff ORIs. Consistent with the known role of the ecdysone receptor as a transcription factor, the complex can then induce the transcription of small non-

coding RNAs (ncRNAs) that are complementary to the DNA puff origin. Subsequently, the small ncRNAs can associate with ORC or other replisome components to recruit ORC to DNA puff ORIs complementary to their sequence. A role for ncRNAs in the recruitment of ORC to replication initiation sites has several published precedents (Mohammad et al. 2007, Norseen et al. 2008, Collart et al. 2011, Ge & Lin 2014). In *Tetrahymena thermophila*, an RNA fragment derived from the terminal 282 nucleotides of 26S rRNA associates with ORC, specifically recruiting ORC to its complementary sequence at the rDNA origin on the 21kb rDNA minichromosome, resulting in 9000 rounds of replication of the minichromosome (Mohammad et al. 2007). The genome of the Epstein-Barr virus (EBV) encodes the protein EBNA1, which binds to EBV plasmid origins and recruits ORC through interactions mediated by small, G-rich ncRNAs (Norseen et al. 2008). A class of ncRNAs called Y RNAs, which are 70-120 nucleotide-long stem-loop RNAs, interact with ORC at replication origins in *Xenopus laevis*, and are required for DNA replication (Collart et al. 2011). In *Sciara*, preliminary northern blot experiments, using the II/9A DNA puff 1kb ORI as a probe, identified a small 200 nt RNA in total RNA complementary to the 1kb ORI. It is a tantalizing possibility that this 200 nt RNA interacts with the replication machinery to guide ORC specifically to the II/9A ORI. This finding has not since been confirmed or extended to other DNA puffs, and ongoing work to follow up on this finding are presented in this thesis.

DNA re-replication events resulting from deregulation of DNA replication initiation can lead to genomic instability and subsequent cellular dysfunction, and can thus promote tumorigenic pathways. Two convenient model systems exist for experimental investigation of DNA replication initiation mechanisms that can lead to DNA amplification: *Drosophila* amplicon in follicle cells and *Sciara* DNA puffs. In *Sciara*, accumulating evidence suggests that the ecdysone receptor is the amplification trigger for II/9A. Using qPCR analysis, this thesis shows that the injection of ecdysone induces amplification at all candidate DNA puffs identified from the recently-assembled *Sciara* genome, suggesting that ecdysone and the ecdysone receptor may be the amplification triggers for all DNA puffs. Additionally, this thesis outlines ongoing experiments addressing the hypothesis that ecdysone induces transcription of ncRNAs complementary to DNA puff ORIs that recruit ORC to the DNA puff ORIs for DNA amplification.

2. Ecdysone-Induced DNA Amplification

2.1 – Ecdysone-induced DNA amplification at DNA puff II/9A is consistent with previously-determined amplification levels

Previous ecdysone-injection experiments in *Sciara* by Foulk et al. (2006) quantified the induced DNA amplification at the II/9A locus 24 hours post-injection using quantitative PCR (qPCR). This was possible due to the availability of the DNA sequence of locus II/9A, which is the most well-characterized locus in the *Sciara* genome. At 24 hours post-injection of early-eyespot larvae, Foulk et al. (2006) measured an approximate 16-fold amplification at II/9A. I extended this approach to study a total of 14 DNA puff loci in the *Sciara* genome. As an initial validation of the ecdysone-injection and qPCR methods used in the experiments for this thesis, I quantified DNA amplification at the II/9A locus 24 hours post-injection of Early-Eyespot larvae. Eyespots provide a convenient phenotypic marker to stage the larvae. Younger larvae which do not have eyespots cannot be accurately staged because the developmental timeframe during which larvae do not yet develop eyespots is too long. Therefore, Early-Eyespot larvae are the youngest pre-DNA amplification-stage larvae that can be staged with accuracy. Following the injection parameters by Foulk et al. (2006), 32 nl of 1 mg/ml 20-hydroxyecdysone (hereafter 'ecdysone') in 50% EtOH were posteriorly-injected in the hemolymph of each larva. As a control, a separate population of age-matched larvae were each injected with 32 nl of 50% EtOH lacking ecdysone. Injected larvae were incubated in a 21°C incubator for 24 hours with sufficient food.

After 24 hours, salivary gland pairs from five larvae were dissected from both ecdysone-injected and control ethanol-injected larvae, serving as one biological replicate. A total of three biological replicates were tested for both ecdysone and ethanol injections. Genomic DNA was extracted from each biological replicate and used as a qPCR template with primers specific to the II/9A amplicon locus and primers specific to a locus that is not amplified during development to serve as an internal control (additional details in Methods). Consistent with the data by Foulk et al. (2006), ecdysone injection resulted in 15.4-fold enrichment of DNA at DNA puff locus II/9A (Figure 7). Normally, this level of DNA amplification is seen at the 14x7 stage, several days after the Early-Eyespot stage. Therefore, the salivary gland chromosomes of ecdysone-injected larvae undergo DNA amplification at DNA puff locus II/9A several days before it is normally expected to occur.

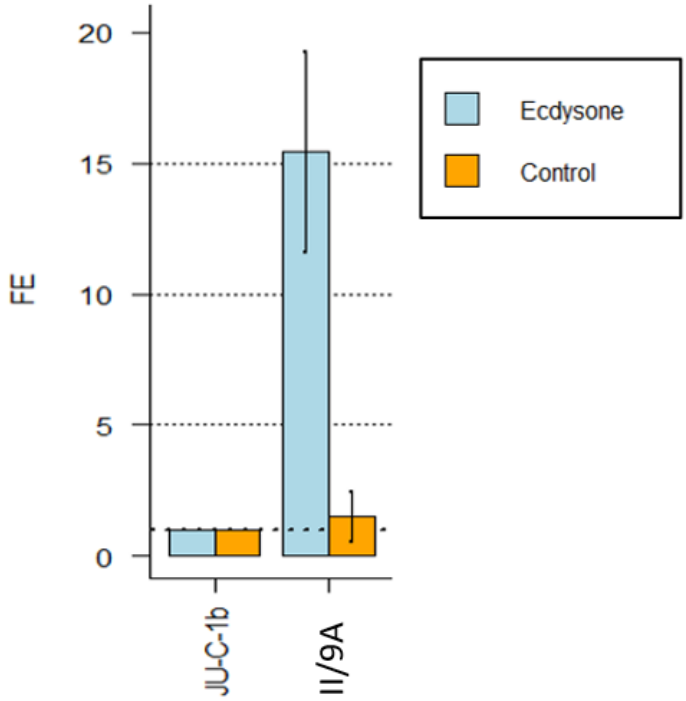

Figure 7 – Ecdysone injection of Early-Eyespot stage larvae induces approximately 15-fold enrichment (FE) at the II/9A locus 24 hours after injection, consistent with experiments by Foulk et al. (see Figure 6). As reference, a 16-fold enrichment corresponds with 4 additional rounds of re-replication ($2^4 = 16$). Ecdysone does not induce premature amplification in larvae injected with control 50% EtOH, the solvent used for ecdysone. FE was calculated using the $\Delta\Delta Ct$ method (additional details in Methods), which uses both an internal normalizer locus and an external normalizer. JU-C-1b is an internal normalizer locus that does not amplify during development. All bars represent the means of three biological replicates. Adult Sciara DNA which does not undergo amplification, was used as the external normalizer.

2.2 – Ecdysone induces accelerated phenotypic changes in body and eyespot shape, but not in eyespot number

Ecdysone-injected larvae exhibited premature Edge-Eye-stage phenotype 24 hours post-injection, also consistent with observations by Foulk et al. (2006) (Figure 8). This includes shortening of the larval body, increased body opacity, and pronounced ribbing of the cuticle. Ethanol-injected larvae did not undergo any phenotypic changes. The Edge-Eye stage was prematurely induced in the ecdysone-injected larvae several days earlier than would be expected in the normal developmental timeline of *Sciara* larvae. During normal development, the II/9A locus of the salivary gland polytene chromosomes reach 17-fold amplification at the 14x7 stage (Figure 5) but has been found to reach as high as 32-fold at the Edge-Eye stage (unpublished qPCR data from the Gerbi Lab). Thus, in ecdysone-injected larvae, an Edge-Eye phenotype was prematurely induced but DNA amplification was only induced up to 14x7 stage levels, not to

Edge-Eye stage levels.

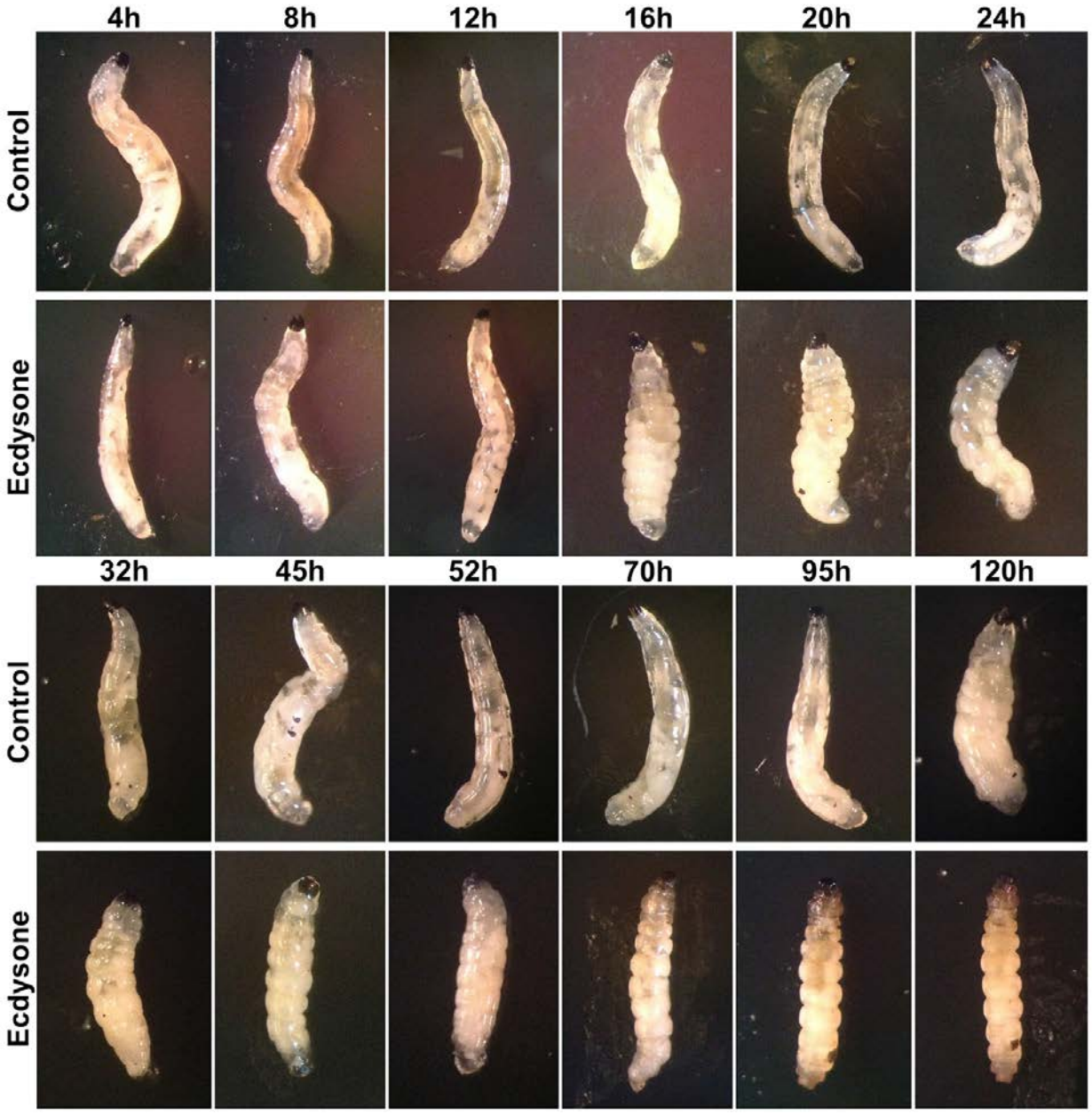

Figure 8 – Ecdysone injection prematurely induces phenotypic changes. The Edge-Eye phenotype (refer to Figure 4 for normal developmental phenotypes) is prematurely induced starting at 16 hours post-ecdysone injection. After ~45-52 hours post-injection, phenotypes of ecdysone-injected larvae diverge greatly from

any phenotype seen during normal development. Ecdysone-injected larvae have never been observed to pupate successfully or to develop into viable adults. Larvae injected with control 50% EtOH develop normally.

Throughout the research history of *Sciara*, eyespot number and morphology was used to identify the developmental stage of *Sciara* larvae. Larvae are characterized as progressing, in order, through the following stages: pre-eyespot → early-eyespot → 8x4 → 10x5 → 12x6 → 14x7 → Edge-Eye → Drop-Jaw → Pupa (Figure 4). Up until the 14x7 stage, *Sciara* larvae are very mobile, and phenotypic differences between stages is mostly limited to increasing fat body, opacity, and size with increasing stage. However, from Edge-Eye onwards, a dramatic phenotypic shift occurs that involves the shortening and widening of the body, a pronounced ribbing of the cuticle, increased white opaque color, and, most notably, the coalescing and lateral movements of the eyespots. Therefore, 'Edge-Eye' eyespots has typically been considered to follow the 14x7 stage. In other words, the formation of Edge-Eye eyespots is thought to have been dependent on larvae first forming 14x7 eyespots. Interestingly, this was not the case in the 24 hour ecdysone injections. Ecdysone-injected larvae prematurely progressed to the Edge-Eye stage, but without any noticeable increase in eyespot count (Figure 9). In effect, larvae prematurely transitioned from 'Early-Eyespot→Edge-Eye' stages, skipping altogether the intervening 8x4, 10x5, 12x6 and 14x7 stages rather than accelerating through them. This suggests that the developmentally-regulated increase in eyespot count, and the shape of eyespots, are not closely linked

processes as previously thought. Instead, the increase in eyespot count and the transition into Edge-Eye form appear to be two distinct processes such that ecdysone induces the latter, but not the former. According to such a model, ecdysone injection induces the lateral migration of eyespot pigment granules as is characteristic for Edge-Eye eyespot shape using whichever number of eyespots are present at the time of injection. Ecdysone injections using 8x4 stage larvae also produced the same results, where the eyespots prematurely coalesced into the Edge-Eye eyespot shape, altogether skipping the 10x5, 12x6, and 14x7 stages (data not shown).

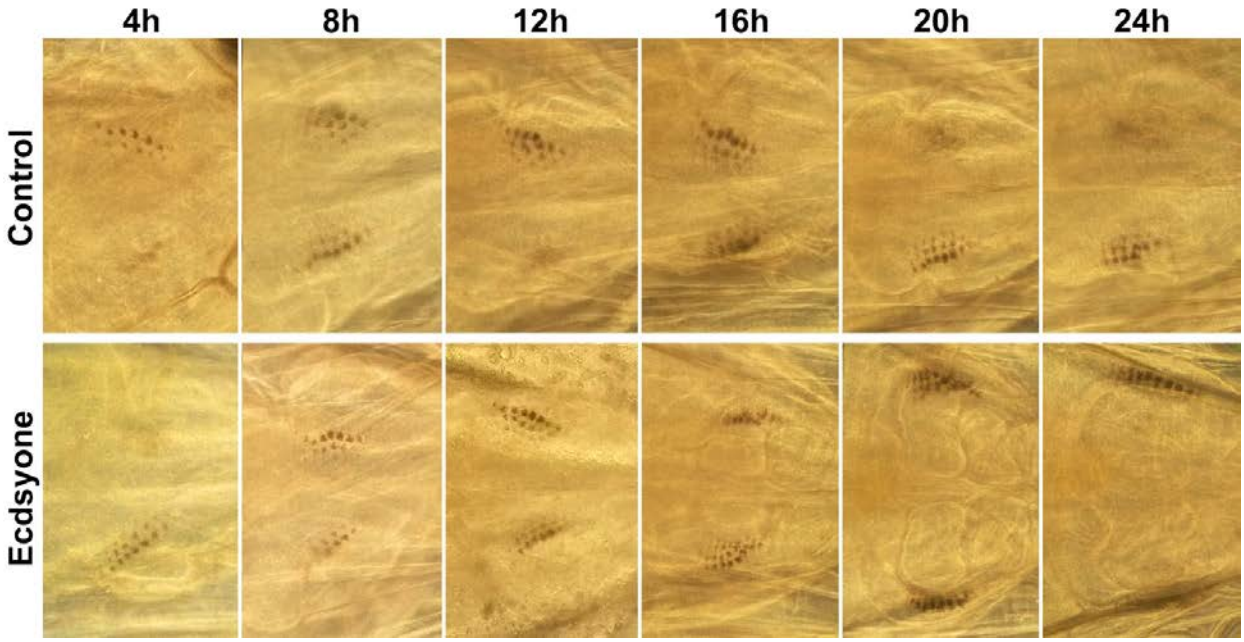

Figure 9 – Ecdysone-induced changes in larval eyespot phenotype. Normally, the number of eyespots in Sciara larvae continue to increase until the 14x7 eyespot arrangement (see Figure 1), at which point the eyespots coalesce and migrate

laterally to form the 'edge-eye' shape. The eyespots in ecdysone-injected larvae are directly induced into the 'edge-eye' shape and migrate laterally, completely skipping rather than accelerating through the increase in the number of eyespots. Eyespots of larvae injected with control 50% EtOH develop normally and do not prematurely coalesce or migrate laterally.

2.3 – Ecdysone induces DNA amplification at all candidate DNA puff loci identified in the Sciara genome assembly

Completion of the genome assembly of *Sciara*, which was done in parallel with this project by Dr. John Urban in the Gerbi Laboratory, identified 14 candidate DNA puff amplicons across the four chromosomes. Candidate DNA amplicons were identified as regions with elevated copy numbers relative to the rest of the genome in the sequencing data. These 14 candidate DNA puffs were arbitrarily named JU-T-1 through JU-T-14 (JU= John Urban, T=Target amplicon) until their sequences could be mapped to the chromosomes using Fluorescence *in situ* Hybridization (FISH), which would confirm that they colocalize cytologically with DNA puffs. Prior to the genome assembly, 18 DNA puff amplicons were already identified and mapped to the *Sciara* salivary gland polytene chromosomes purely by cytological experiments. It is likely that the set of 14 candidate DNA amplicons identified through the genome assembly is a subset of the previously-identified 18 DNA amplicons. Indeed, this was found to be the case in FISH experiments done in parallel by Dr. Yutaka Yamamoto of the Gerbi Laboratory. Probes generated from the candidate DNA puff sequences JU-T-1, JU-T-2, JU-T-5, JU-T-8, JU-T-9,

and JU-T-10 hybridized to known chromosomal locations of DNA amplicons II/9A, II/2B, II/11A, X/11B, III/11B, and IV/5C respectively (Table 1). FISH experiments for the remaining 8 candidate amplicons are still in progress, but are likely to also correspond to known DNA amplicon loci. In addition to the qPCR experiments by Foulk et al. (2006) demonstrating that ecdysone can induce premature DNA amplification at II/9A, earlier radiological studies showed increased DNA synthesis at the 18 DNA puffs in response to ecdysone injection, suggesting that premature DNA amplification can be induced in all DNA amplicons (Crouse 1968). Therefore, an additional method of validating whether the 14 candidate DNA amplicons identified in the genome assembly are real DNA amplicons is to determine whether they are responsive to ecdysone, i.e. whether ecdysone induces premature DNA amplification and the candidate loci. With the completed genome now available to design qPCR primers for all candidate DNA puffs, I extended qPCR analysis of salivary gland 24 hr post-ecdysone-injection to the remaining 13 candidate DNA puffs. Ecdysone induced premature DNA amplification at all thirteen of these candidate amplicons, supporting the hypothesis that the fourteen candidate amplicons are a subset of the previously-identified 18 amplicons (Figure 10, top). Statistical significance, assessed as $p<0.05$ for a two sample t-test testing the null hypothesis difference in the fold-enrichment means in ecdysone and ecdysone-injected samples is zero, was not reached for all DNA amplicons in the ecdysone sample when the amplicons were tested separately (Supplementary Table 2). However, the combined observations that all 14 candidate amplicons underwent amplification in response to ecdysone was extremely statistically significant

(2.56e-20). Taken together, the finding that all candidate DNA amplicons can be induced by ecdysone validates these sequences as true DNA amplicons and not false positives from the genome sequence, and, more importantly, adds to accumulating evidence that ecdysone and the ecdysone receptor are amplification triggers at DNA puff loci.

Assigned Candidate DNA Puff Name	Corresponding chromosomal position determined by FISH
JU-T-1	II/9A
JU-T-2	II/2B
JU-T-5	II/11A
JU-T-8	X/11B
JU-T-9	III/11B
JU-T-10	IV/5C

Table 1 – Chromosomal positions of candidate DNA puffs as verified by FISH. 14 candidate DNA puffs were identified in the Sciara genome assembly and named JU-T-1 through JU-T-14. Using the sequences of these candidate DNA puffs, FISH probes were generated to map the candidate DNA puff sequences to the chromosomes and determine which of the cytologically known 18 DNA puffs a particular candidate DNA puff corresponds to.

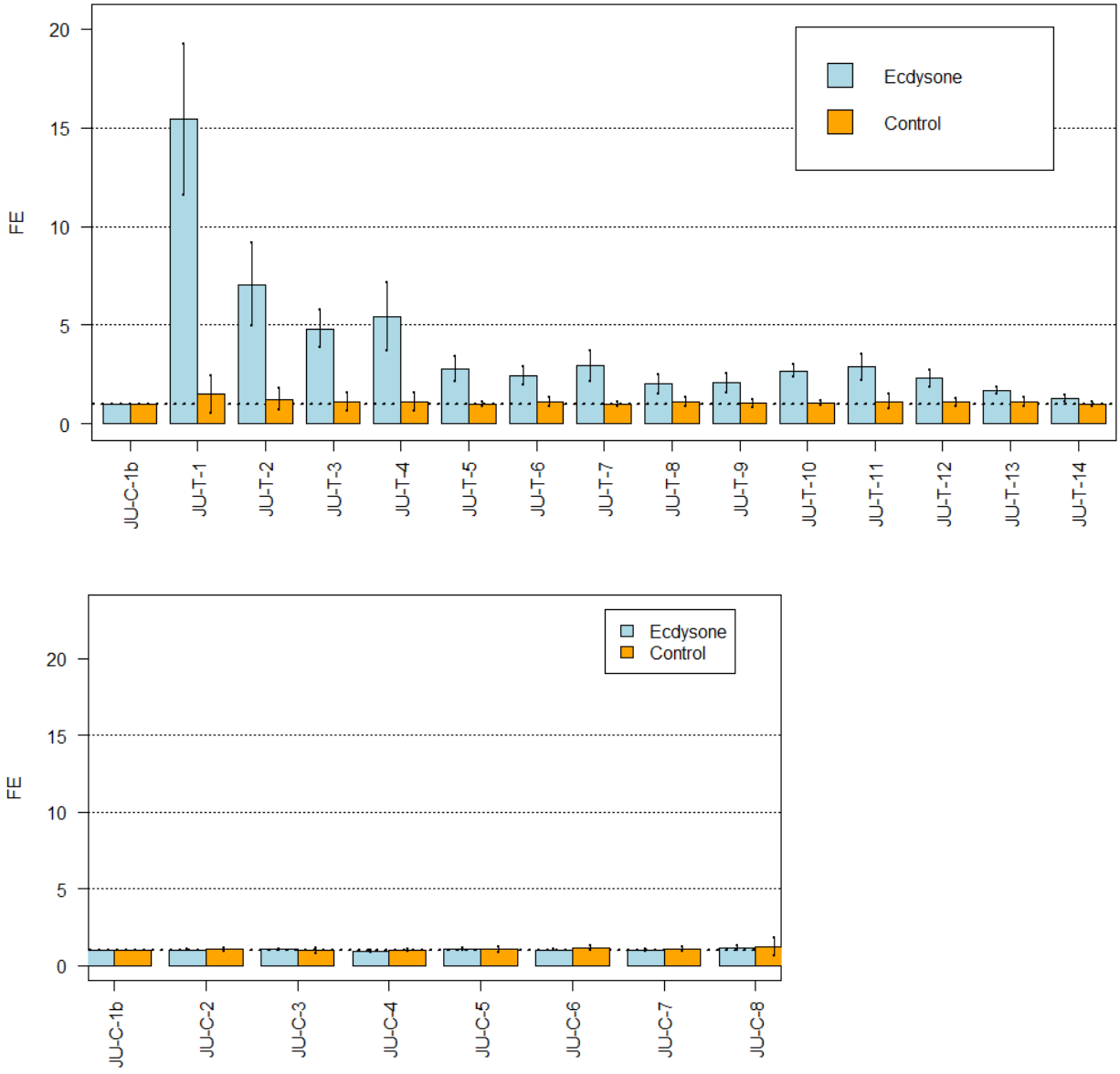

Figure 10 – DNA amplification 24 hours post-ecdysone injection. (Top) Ecdysone injections induce DNA amplification at all fourteen candidate DNA puffs identified in the genome assembly. The p-value for two-sample Student's t-test is 2.56e-20 (see Supplementary Table 2). JU-C-1b is an internal normalizer locus that does not amplify during development and is located on the same contig as JU-T-1 (II/9A) in the genome assembly (Bottom) Ecdysone injections do not induce DNA

amplification at control loci (JU-C-1 through JU-C-8) that are not normally amplified during development. FE=fold enrichment. Graphs display the mean FE of three biological replicates.

The specificity of ecdysone-induced DNA amplification at the candidate DNA puff loci is demonstrated by the observation that ecdysone does not induce DNA amplification at loci that do not normally amplify during development (Figure 10, bottom). The control loci used in the analyses are named JU-C-1 through JU-C-8. JU-C-1(b) is the control locus used as the internal normalizer for all qPCR analyses. These control loci are located on the same contigs in the genome assembly as the amplicon loci. For example, JU-C-1 is located on the same contig as the amplicon JU-T-1 (II/9A), JU-C-2 is located on the same contig as the amplicon JU-T-2, etc.

Although all 14 candidate amplicons were ecdysone-responsive, the magnitude of ecdysone-induced DNA amplification varied greatly between amplicons. Amplification was greatest at amplicons II/9A and II/2B, where ~15.4 fold and ~7 fold enrichment was observed 24 hours post-injection. Fold enrichment at the remaining amplicons varied between ~0.5 and 5.5. The magnitude of ecdysone-induced DNA amplification is correlated with the timing of amplification in normal development (unpublished data from Dr. John Urban). For example, II/9A is the earliest locus to begin amplification, while smaller amplicons such as JU-T-12-14 are the latest to begin amplification during normal development. The mechanisms that dictate the order in which amplicons begin amplification during normal development are not yet known. Given that the relative magnitudes of each

amplicon is conserved in normal amplification and in ecdysone-induced amplification, it is likely that factors other than ecdysone and EcR play important mechanistic functions. For example, some factors may enhance ORC recruitment and amplification at II/9A. This could be at the amplicon sequence level such that amplification factors have higher affinity for a motif in II/9A relative to other puffs.

2.4 – Ecdysone-induced DNA amplification 48 hours post-injection is lower than 24 hours post-injection, possibly due to ecdysone-induced salivary gland histolysis and DNA degradation

During normal development, qPCR data by graduate student Audrey Lee suggests that amplification at the II/9A locus reaches ~17 fold at the 14x7 stage (Figure 5), and increases up to ~32 fold at the Edge-Eye stage, which is the highest-fold amplification seen during development (Figure 11). However, 24 hours post ecdysone-injection when larvae prematurely progressed to the Edge-Eye stage, amplification at the II/9A locus was only ~17 fold, as stated earlier.

This suggested the possibility that ecdysone-induced acceleration of external larval morphology might be faster than the acceleration of DNA amplification.

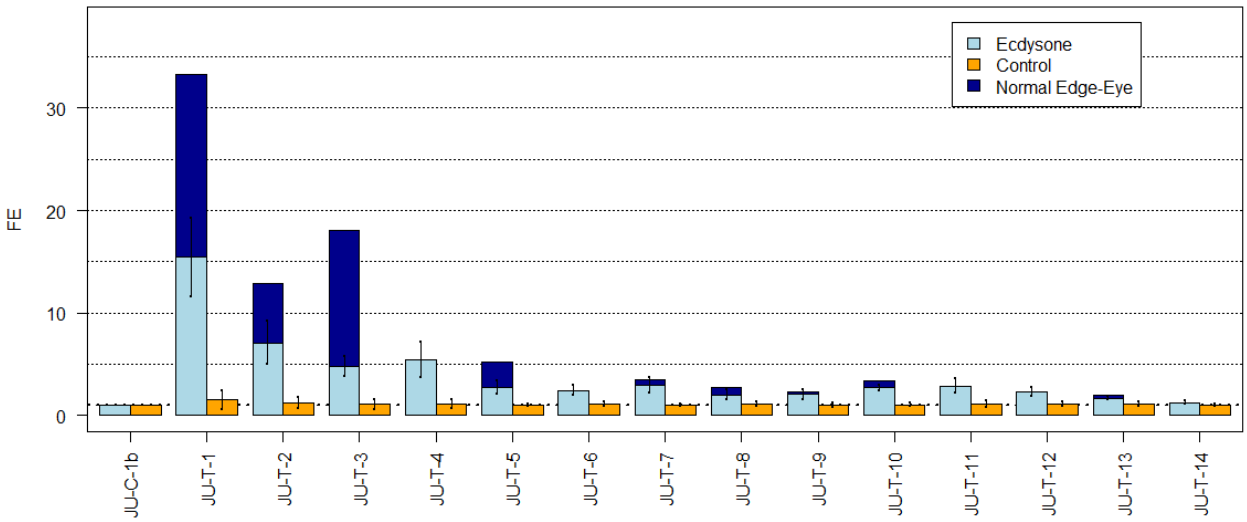

Figure 11 – DNA amplification 24 hours post-ecdysone injection, superimposed with DNA amplification levels normally seen (i.e. not from injection experiments) during Edge-Eye stage. The difference between amplification levels in normal Edge-Eye stage and 24 hr ecdysone-induced amplification levels suggested that ecdysone-induced amplification levels might reach higher levels past 24 hours.

Therefore, I explored the possibility that ecdysone-induced DNA amplification could reach higher fold-enrichment past 24 hours. I performed qPCR analysis on salivary gland DNA extracted 48 hours post-ecdysone injection. I hypothesized that, after 24 hours, ecdysone-induced DNA amplification would reach levels normally seen during development at Edge-Eye stage (~32 fold amplification). The qPCR data showed completely opposite results. Higher levels of DNA amplification were not reached at any amplicon locus after 48 hours (Figure 12). At every

amplicon locus, ecdysone-induced DNA amplification at 48 hours post-injection was lower than or not significantly different from DNA amplification at 24 hours post-injection. The greatest decrease in fold-amplification was observed at amplicons that showed the greatest amplification at 24 hours – amplicons II/9A, II/2B, and JU-T-3 (JU-T-3 is not yet mapped to any chromosome). The observation that the most amplified amplicons show the greatest decrease in amplification from 24 to 48 hours post-injection suggests that this decrease is attributable to a DNA breakdown mechanism.

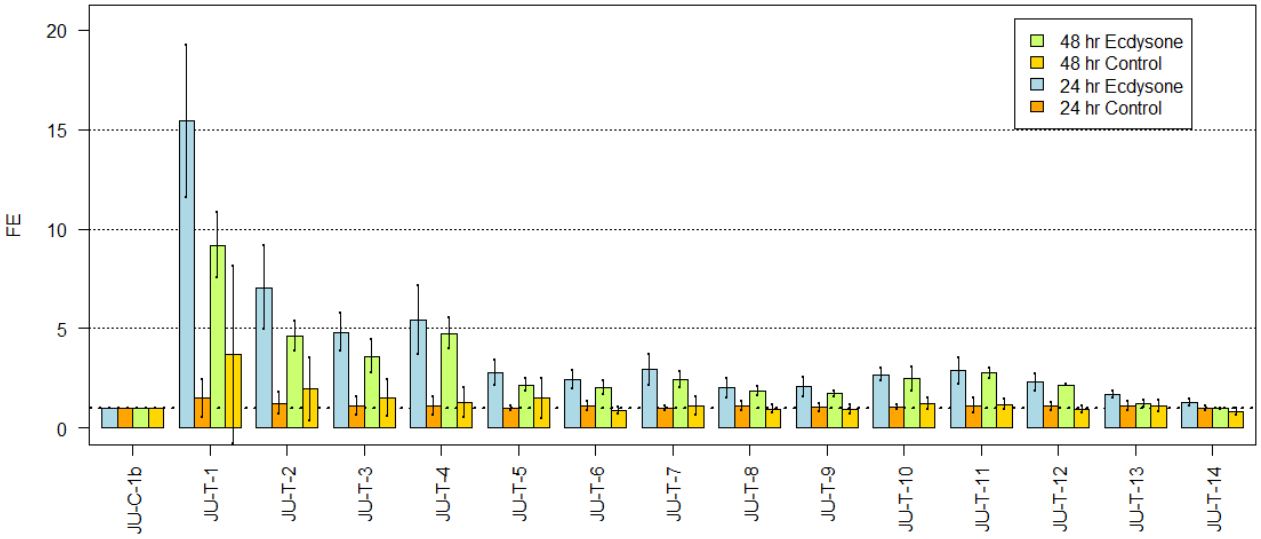

Figure 12 – DNA amplification 48 hours post-ecdysone injection, shown alongside DNA amplification levels 24 hours post-ecdysone injection from Figure 10. Amplification levels at all amplicons decrease from 24 to 48 hours, likely due to ecdysone-induced histolysis and DNA degradation that occur in parallel with DNA amplification.

During normal larval development, larval tissues, such as salivary glands, undergo histolysis, the regulated breakdown of tissues, during the larva-pupa

transition. Histolysis is regulated by ecdysone, and causes the disintegration of larval tissues. Specifically, the amount of DNA in Dipteran salivary glands has been shown to decrease in late prepupal larval stages due to increased DNase activity (Laufer et al. 1967, Farkas & Mechler 2000). Histolysis of salivary glands during normal *Sciara* development avoids the potential genomic instability from DNA amplification in adult tissues where the cells undergo mitosis. It is possible that the injected ecdysone is prematurely accelerating the onset of histolysis, leading to degradation of amplicon DNA and the observed decrease in amplification levels at 48 hours post-injection. Consistent with known phenotypic changes associated with histolysis in Dipteran salivary glands, salivary glands dissected from larvae 48 hours post-ecdysone-injection were shrunken, more frail, and more withered than salivary glands from control larvae (data not shown). Externally, ecdysone-injected larvae show a premature Edge-Eye/Drop-Jaw stage phenotype between 45-52 hours (Figure 8). However, the salivary glands in ecdysone-injected larvae still appear more degraded than salivary glands from normal Edge-Eye/Drop-Jaw larvae. These phenotypic observations suggest that histolysis of salivary glands in ecdysone-injected larvae is induced at a higher rate than the changes in external morphology, and that this is responsible for degrading DNA sometime after 24 hours post-injection, leading to the observed decrease in DNA amplification from 24 to 48 hours post-injection.

2.5 – Ecdysone-induced DNA amplification at 16 hours post-injection is lower than at 24 hour post-injection

In order to follow the developmental profile of accumulation of amplified DNA after ecdysone injection, I also examined the qPCR analysis for 16-hour post-ecdysone injection larvae. This time point was chosen because it is the earliest time point following ecdysone injection at the Early-Eyespot larvae undergo premature phenotypic changes into the Edge-Eye phenotype. Ecdysone-induced DNA amplification was observed at the amplicons at 16 hour post-injection (Figure 13). However, the amplification levels at 16 hours post-injection were all lower than at 24 hour post-injection. This indicates that the level of amplified DNA continued to increase after 16 hours. It is possible that the amplification levels peaked sometime between 24 hours and 48 hours, but the data from these three time points suggests that the 24 hour qPCR data is a good estimate of the peak levels of ecdysone-induced DNA amplification across all of the puffs.

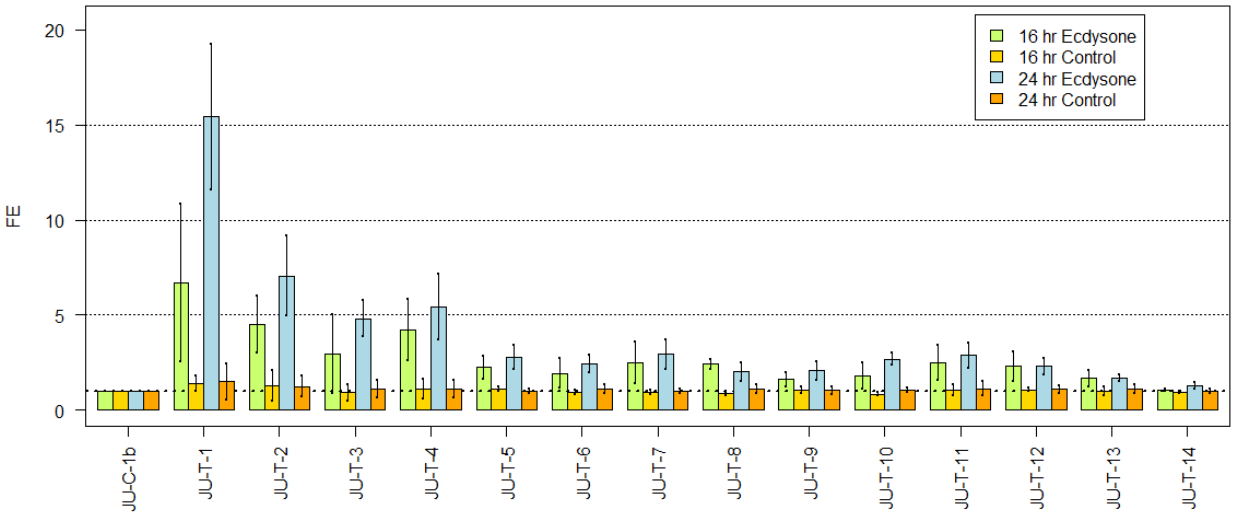

Figure 13 - DNA amplification 16 hours post-ecdysone injection. 24 hour data from Figure 10 plotted for comparison. Ecdysone-induced DNA amplification is in progress at 16 hours post-injection, but is lower than that seen at 24 hours.

Ecdysone-induced DNA degradation therefore starts to exceed the rate of DNA amplification sometime between 24-48 hours post-injection, but not between 16-24 hours post-injection.

2.6 – Ecdysone-induced larvae undergo severely abnormal phenotypic changes after 45-52 hours post-injection and fail to pupate

No ecdysone-injected larva has been observed to survive until adulthood. Although ecdysone-injected larvae do prematurely develop a normal Edge-Eye phenotype, any further changes in phenotype differ drastically from normal development (Figure 8, Figure 14). In normal development, after the Edge-Eye stage, larvae will progress to the Drop-Jaw stage during which the jaw will physically detach from the body as the larvae transition to the pupal form (Figure 4). In contrast, the jaws of ecdysone-injected larvae remain physically attached to their body until their death (Figure 8, Figure 14). The posterior body begins to develop pigmented segmentation patterns while the anterior end develops partial pupal-like structures, such as legs and wings. Thus, ecdysone-injection leads to complete failure to pupate and eventual death, after premature but incomplete development of certain structures associated with the pupal stage.

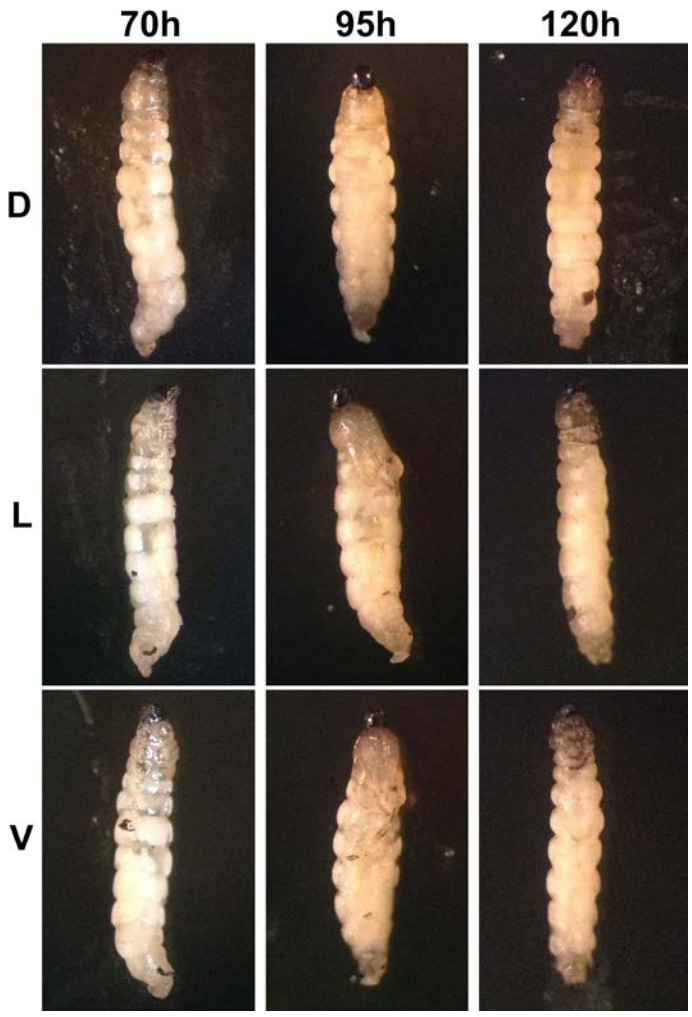

Figure 14 – Dorsal (D), lateral (L), and ventral (V) view of abnormal development of ecdysone-injected larvae after 70 hours post-injection. Ecdysone-injected larvae prematurely develop the Edge-Eye phenotype at 16-24 hours (Figure 8) and maintain the Edge-Eye phenotype until approximately 45-52 hours. After 52 hours, severe abnormal phenotypic changes occur. Certain components of the larval phenotype are retained, eg. elongated body and jaw attached to head, but certain partial pupal structures also unsuccessfully form, such as eyes, wings, legs

2.7 – Conclusions from ecdysone injections

The responsiveness of all 14 candidate DNA puffs accomplished several goals. First, these experiments provide strong quantitative evidence supporting the hypothesis that ecdysone, and by extension the ecdysone receptor, is the amplification trigger for all DNA puffs in *Sciara*. The observations that the relative levels of ecdysone-induced amplification across all candidate DNA puffs is nearly identical to that seen during normal development further supports this hypothesis. During normal development, amplification levels at II/9A increase from ~17 fold to ~32 fold, as measured by qPCR, as larvae transition from 14x7 to Edge-Eye stage. However, amplification levels in ecdysone-injected larvae do not exceed the ~17 fold threshold despite prematurely transitioning to an Edge-Eye body phenotype. The ecdysone-injection qPCR results give amplification levels for DNA puff II/9A that is the same as found by quantitative Southern blots (Wu et al. 1993).

3. Finding a Small ncRNA Complementary to the 1kb II/9A ORI

3.1 - Does a small ncRNA promote ORC selectivity at DNA puff II/9A?

The results from the injection experiments showing that all candidate DNA puffs are ecdysone-responsive definitively implicate ecdysone in the DNA amplification mechanism. However, they do not provide much insight into a mechanistic role. One hypothesis is that ecdysone induces binding of EcR to the EcRE upstream of ORIs, activating transcription of small non-coding RNAs (ncRNAs) complementary to DNA amplicon ORIs. These small RNAs can then associate with components of the replisome to selectively guide ORCs to amplicon ORIs through complementary base pairing. Such a hypothesis would be consistent with EcR's known role as a transcription factor. Additionally, the detailed map of the II/9A amplicon locus (Figure 3) reveals an EcRE upstream of the ORC binding site in the 1kb ORI for the II/9A amplicon. The *Sciara* isoform of the EcR prematurely localizes to DNA puff sites in response to ecdysone injections (Liew et al. 2013). A role for small ncRNAs in facilitating ORC-ORI interactions has been documented multiple times, such as the rDNA replication mechanism in *Tetrahymena thermophila*, plasmid replication in cells infected with the Epstein-Barr Virus, and DNA replication in *Xenopus laevis* during the midblastula transition (Mohammad et al. 2007, Norseen et al. 2008, Collart et al. 2011). In *Sciara*, preliminary Northern blots by Dr. Janell Johnson from the Gerbi Laboratory, using a dsDNA probe generated from the 1kb ORI of the largest *Sciara* amplicon II/9A, showed a ~200 nt RNA complementary to the ORI that appears in larval salivary gland cells during DNA amplification stages. The work described in this section

details methods to validate this finding and determine whether DNA amplification at the II/9A amplicon origin may involve a small RNA mechanism.

3.2 – Northern blot probing for small RNAs complementary to the II/9A 1kb ORI

We are currently in the process of validating the results of the preliminary Northern blot by Dr. Janell Johnson by repeating the Northern blot, with a few adjustments, detailed below:

3.2(i) – RNA sample preparation

To validate the preliminary results with my own Northern blot, I wanted to enrich my salivary gland RNA sample for RNA smaller than ~250 nt by depleting larger RNAs from the sample. Initially, I tested whether Agencourt AMPure XP Beads from Beckman Coulter could be used to size-select RNA from a fragmented total RNA sample by adjusting the volume of the AMPure bead solution added to the total RNA sample. This was unsuccessful; the beads could not size select for small RNAs.

Based on email correspondence with Dr. Jason Wood from the Helfand Laboratory, the next approach was to use the mirVana miRNA Isolation kit, which allows for isolation of RNAs smaller than ~200 bases. The kit was initially tested on total RNA isolated from whole *Sciara* larvae. The kit did indeed successfully separate small RNAs from large RNAs (Figure 15). However, the size selection boundary was slightly too low for enrichment of 200-250 nt RNAs. The upper size

limit of the small RNA fraction is dependent on the final concentration of ethanol in the RNA sample before passing the sample through the first purification column. The kit manual suggests a 25% EtOH concentration to bind RNAs larger than 200 nt onto the column and purify <200 nt RNAs from the flow-through in subsequent steps. I adjusted this initial ethanol concentration and found that 18% EtOH was optimal for purifying small RNAs for use in the Northern blot. The effects of changing the ethanol concentration on the size selection boundary can be seen in (Figure 15) based on the change in intensity of the 5.8S and 5S rRNA bands in the small RNA sample relative to the large RNA sample.

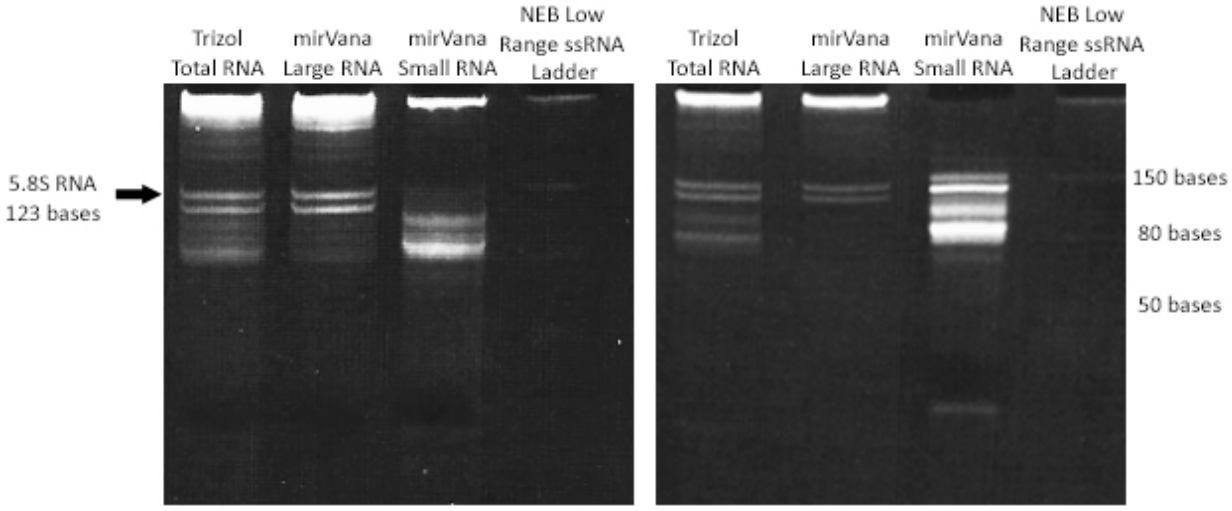

Figure 15 – Comparison of adjusted size selection boundaries of small and large RNA fractions isolated from whole Sciara larvae (n=25) using the mirVana miRNA Isolation Kit. (Left) mirVana manual was followed exactly, using 25% EtOH in the first column purification step. 5.8S RNA band (123 bases) is indicated to provide an estimate of the size boundary between small and large

fractions. Total RNA extracted using Trizol is shown as reference. (Right) Ethanol concentration for the first column purification was decreased to 18% to increase the size selection boundary between small and large RNA fractions using the mirVana kit. The increased size boundary is reflected in the increased intensity of the 5.8S RNA band in the small RNA fraction relative to the large RNA fraction, compared to the samples isolated using 25% EtOH. Loading conditions were identical for both gels. Samples were run on 15% Urea PAGE.

With the RNA size selection method optimized, I used the mirVana miRNA kit to isolate small RNA samples from each of the following stages: Early-Eyespot, 8x4, 10x5, 12x6, and 14x7. For each of these five stages, 130 pairs of salivary glands were dissected for a single RNA sample, for a total of five RNA samples.

3.2(ii) – Probe preparation

The probe used for the preliminary Northern blot by Dr. Janell Johnson was prepared from a double-stranded DNA fragment of the 1kb II/9A ORI, and so it is not known which strand is complementary to the detected 200 nt RNA. The probes that I will use in my Northern blot will instead be prepared from single-stranded RNA (ssRNA). To prepare ssRNA probes, I cloned a 1kb II/9A fragment and an 88 bp 5.8S rDNA fragment into pCR-Blunt II-TOPO vectors. The 5.8S rDNA was selected as the control to prepare the ssRNA probe because the size of 5.8S rRNA lies within the size isolated in the small RNA fraction using the mirVana miRNA kit. The TOPO vector has a T7 promoter and SP6 promoter located at opposite ends

of the insertion site in opposite orientations, allowing for *in vitro* transcription of both strands of the insert. ssRNA will be transcribed from both strands for both the 1kb II/9A fragment and the 5.8S rDNA fragment and radiolabeled with [α-32P]-UTP. Probing the larval salivary gland small RNA samples with ssRNA II/9A probes from both strands will reveal which strand encodes the detected 200 nt RNA. The 5.8S probe transcribed from the antisense strand will serve as a positive control, while the 5.8S probe transcribed from the sense strand will serve as a negative control.

3.3 – Ongoing work and future directions

At the time of submission of this thesis, the Northern blot experiments described above are still in its preparatory stages. Small RNA samples from all five stages have been collected, and the 5.8S rDNA and 1kb II/9A ORI fragments have been cloned in the TOPO vectors for *in vitro* transcription and subsequent radiolabeling for use as probes. The rest of the procedure has not yet been completed, and so optimization details for the rest of the procedure, and results from subsequent Northern blots between the time of thesis submission and university Commencement, will be included as an amendment to this thesis. The Methods section details parts of the Northern blot procedure that have been completed and optimized and parts that have yet to be completed. If these Northern blots confirm the preliminary finding of a 200nt RNA complementary to the 1kb II/9A ORI, smaller probes generated from regions within the 1kb II/9A ORI can be used to narrow down the position of the 200 nt RNA. Alternatively, a DNA pulldown assay using the 1kb II/9A ORI to pull down complementary RNA can be

used to isolate the 200 nt ncRNA for sequencing. Once the sequence of the 200 nt RNA is determined, an RNA pulldown assay can be used to purify associated proteins, and these proteins can be identified using mass spectrometry. If associated proteins are known components of the replisome, it will support the hypothesis that small ncRNAs complementary to DNA puff origins help to selectively recruit ORC to amplicon origins. The identification of proteins that are not known to be involved in DNA replication would also be an important finding, and would help elucidate novel replication mechanisms used during DNA amplification.

4. Discussion

The experiments detailed in this thesis add to a growing body of evidence supporting the hypothesis that ecdysone and the ecdysone receptor may be the amplification trigger for all DNA puff amplicons in the late 4th instar larval salivary gland chromosomes in *Sciara coprophila*. Previously, tritiated thymidine labelling experiments qualitatively showed premature and disproportionately increased DNA synthesis at all DNA puff loci in response to ecdysone injection (Crouse 1968). Additionally, quantitative data from qPCR experiments showing that ecdysone can prematurely induced DNA amplification was only available for DNA puff II/9A, the largest and earliest-appearing puff (Foulk 2006). At the time, only the II/9A sequence was available for qPCR primer design. However, recent completion of the *Sciara* genome assembly allowed for the identification of fourteen candidate DNA puffs across the genome, six of which have been cytologically mapped to known morphological DNA puffs. Using the genome data, qPCR primers could be designed for all candidate DNA puff loci. In this thesis, this new toolbox was used to extend the quantitative analyses from the single II/9A locus to fourteen amplicon loci, confirming that ecdysone can prematurely induce DNA amplification at all candidate amplicon loci in pre-amplification stage larvae. Additionally, ecdysone-induced DNA amplification was analyzed at three time-points, 16 hr, 24, hr, and 48 hr, which has not been done previously.

In normal development, the DNA amplification level at the II/9A locus increases to approximately 17 fold-enrichment, or approximately 140 000 C

(Figure 5), by the 14x7 stage (Gerbi et al. 1993). The ecdysone-induced DNA amplification at the II/9A locus at 24 hours post-injection (Figure 7) is consistent with these observations. The same data by Gerbi et al. (1993) (Figure 5), measured using quantitative Southern blot, suggests that DNA amplification levels stay constant at ~17 fold past the 14x7 stage into Edge-Eye stage. However, recent qPCR data by graduate student Audrey Lee, using the same primers as the qPCR experiments in this thesis, show that the DNA amplification level at the II/9A locus increases to ~32 fold at the Edge-Eye stage (Figure 11). The discrepancy between the recent qPCR data by Audrey Lee and the data by Gerbi et al. (1993) may reflect methodological differences between qPCR and quantitative Southern blot analyses. In the ecdysone-injected experiments presented in this thesis, DNA amplification levels did not reach the peak levels observed in Audrey Lee's qPCR data, and was instead more consistent with the Gerbi et al. (1993) data. In fact, amplification levels decreased from 24 hours post-injection to 48 hours post-injection (Figure 12), consistent with observations in the literature that the amount of DNA in Dipteran salivary glands decreases in late pre-pupal larval stages as larval tissues undergo histolysis as part of the larva-pupa transition (Laufer et al. 1967, Farkas & Mechler 2000).

During normal development, the relative levels of DNA amplification is correlated with timing of DNA amplification initiation. For example, II/9A and II/2B, the most amplified DNA puffs, begin amplification earlier than the other puff loci, suggesting that they are more amplified in part because they start to amplify earlier. However, the ecdysone injection data suggest that additional factors determine the

relative amplification levels between puffs. If ecdysone and the ecdysone receptor are amplification triggers, then during the ecdysone injection experiments, this trigger would have been administered synchronously at all puffs. Yet, the relative levels of DNA amplification between puffs are still drastically different 16 hours post-injection (Figure 13). This suggests that additional unknown molecular mechanisms promote ORC recruitment and ORI re-firing at the most-amplified puffs, or suppress ORI re-firing at the less-amplified puffs, or a combination of both. This is supported by the observation that amplification does not increase at the smaller DNA puffs between 16 and 24 hours post-injection, while amplification levels at II/9A and II/2B continue to increase by ~8-fold and ~3-fold respectively (Figure 13).

Little is known about the mechanism through which ecdysone induces DNA amplification. One hypothesis is that ecdysone binds to EcR and induces the binding of EcR to EcREs adjacent to amplicon ORIs to activate transcription of small ncRNA complementary to the ORIs. These small ncRNAs can then associate with the replisome machinery to guide ORC to the amplicon ORIs through complementary base-pairing. Multiple studies have implicated ncRNAs in DNA replication initiation, including the rDNA replication mechanism in *T. thermophila*, plasmid replication in Epstein-Barr Virus-infected cells, and Y RNA-dependent DNA replication in *Xenopus laevis* during the midblastula transition (Mohammad et al. 2007, Norseen et al. 2008, Collart et al. 2011). Consistent with this hypothesis in *Sciara*, EcRs prematurely localized to DNA puff amplicons in response to ecdysone injection (Liew et al. 2013). Additionally, all candidate amplicons in the

genome assembly contain putative EcREs, although by random chance these sequences can occur at each of the amplicon loci. Therefore, additional work still needs to be done to map the amplification origin in all DNA puffs, and determine whether the putative EcREs are adjacent to these origins, as is the case for II/9A.

Transcriptome data have been generated for *Sciara*, but was done using a poly-A RNA library. A ~200 nt RNA complementary to the 1kb II/9A ORI was not detected in these transcriptome data, so it is possible that the small RNA detected in the preliminary Northern blot by Dr. Janell Johnson is not poly-adenylated. Ongoing work to repeat this Northern blot, using ssRNA probes transcribed from both strands of the 1kb II/9A ORI instead of a dsDNA probe, will determine which strand encodes the 200 nt RNA. Future experiments aimed at sequencing this small ncRNA and identifying any associated proteins will be greatly informative in understanding the mechanism of DNA amplification.

Cumulatively, these data support the hypothesis that ecdysone, acting through its receptor, may be the amplification trigger for all DNA puff amplicons in *Sciara* larval salivary gland chromosomes. Ongoing work will help determine if a small ncRNA is mechanistically involved in recruiting ORC to the II/9A amplification origin, which can then be extended to the remaining DNA puffs.

5. Supplementary Tables

Supplementary Table 1 – Fold enrichment (FE) and standard deviation (SD) values at amplicon and control loci in response to ecdysone injection or control ethanol injection from qPCR data. JU-T-1 through JU-T-14 are candidate DNA puff amplicons identified from the Sciara genome assembly are amplified during larval development. JU-C-1 through JU-C-8 are control loci that are not amplified during development. JU-C-1 was used as the internal normalizer locus in the qPCR analyses, and so by definition, the FE and SD for this locus are 1 and 0 respectively. ND = not determined

	24 hour ecdysone-injected		24 hour control ethanol-injected		48 hour ecdysone-injected		48 hour control ethanol-injected		16 hour ecdysone-injected		16 hour control ethanol-injected	
	FE	SD	FE	SD	FE	SD	FE	SD	FE	SD	FE	SD
JU-T-1	15.420	3.837	1.511	0.946	9.196	1.643	3.679	4.481	6.670	4.139	1.393	0.393
JU-T-2	7.056	2.105	1.235	0.549	4.618	0.768	1.962	1.567	4.511	1.477	1.292	0.820
JU-T-3	4.818	0.959	1.098	0.465	3.616	0.860	1.511	0.936	2.951	2.061	0.921	0.427
JU-T-4	5.421	1.731	1.119	0.447	4.766	0.800	1.272	0.748	4.219	1.623	1.131	0.516
JU-T-5	2.776	0.638	1.002	0.095	2.173	0.325	1.511	1.004	2.239	0.607	1.108	0.123
JU-T-6	2.456	0.468	1.121	0.214	2.031	0.336	0.878	0.155	1.943	0.777	0.957	0.106
JU-T-7	2.940	0.782	0.997	0.123	2.452	0.390	1.101	0.456	2.498	1.073	0.948	0.108
JU-T-8	2.027	0.495	1.106	0.216	1.848	0.228	0.953	0.198	2.422	0.265	0.870	0.093
JU-T-9	2.078	0.487	1.045	0.197	1.727	0.161	0.941	0.224	1.615	0.375	1.058	0.145
JU-T-10	2.697	0.294	1.060	0.128	2.482	0.600	1.225	0.279	1.794	0.681	0.841	0.100
JU-T-11	2.890	0.659	1.140	0.350	2.767	0.264	1.201	0.258	2.515	0.927	1.053	0.283
JU-T-12	2.298	0.411	1.113	0.201	2.164	0.042	0.931	0.157	2.303	0.785	1.084	0.107
JU-T-13	1.701	0.167	1.122	0.225	1.215	0.169	1.142	0.287	1.674	0.444	1.007	0.219
JU-T-14	1.269	0.168	1.015	0.114	0.992	0.024	0.828	0.188	1.069	0.063	0.929	0.048
*JU-C-1	1.000	0	1.000	0	1.000	0	1.000	0	1.000	0	1.000	0
JU-C-2	1.035	0.055	ND	0.127	ND	ND	ND	ND	ND	ND	ND	ND
JU-C-3	1.052	0.058	ND	0.170	ND	ND	ND	ND	ND	ND	ND	ND
JU-C-4	0.932	0.055	ND	0.089	ND	ND	ND	ND	ND	ND	ND	ND
JU-C-5	1.070	0.051	ND	0.176	ND	ND	ND	ND	ND	ND	ND	ND
JU-C-6	1.041	0.055	ND	0.162	ND	ND	ND	ND	ND	ND	ND	ND
JU-C-7	1.013	0.040	ND	0.162	ND	ND	ND	ND	ND	ND	ND	ND
JU-C-8	1.150	0.151	ND	0.581	ND	ND	ND	ND	ND	ND	ND	ND

Supplementary Table 2 – P values based on qPCR data in Supplementary Table 1 from two sample Student's t-tests testing the null hypothesis that the difference in means of fold enrichment value in response to ecdysone injection and control ethanol injection equals zero. The t-test was done for each locus separately. Not all amplicon loci (i.e. JU-T-#) showed statistically significant amplification levels in response to ecdysone (i.e. p<0.05), likely due to low numbers biological replicates (3), but the product of the p-values, which is a measure of the probability that all amplicon loci respond to ecdysone, showed strong statistical significance.

	P-value from two sample t-test		
	24 Hour Injections	48 Hour Injections	16 Hour Injections
JU-C-1b (normalizer)	ND	ND	ND
JU-T-1	0.058595374	0.071484231	0.1605246737
JU-T-2	0.030498279	0.041442202	0.0028033964
JU-T-3	0.038303992	0.050548148	0.1118070867
JU-T-4	0.008031831	0.038240071	0.0066242691
JU-T-5	0.088138283	0.143368385	0.0566912678
JU-T-6	0.003590345	0.013238118	0.0094262927
JU-T-7	0.114104951	0.008913794	0.0889150797
JU-T-8	0.156123086	0.049585646	0.0290959896
JU-T-9	0.068028774	0.010446597	0.0232384021
JU-T-10	0.029703647	0.013641343	0.1123441706
JU-T-11	0.017299873	0.023541280	0.0322563028
JU-T-12	0.029030119	0.001193727	0.0005391169
JU-T-13	0.051291218	0.355626754	0.0343506559
JU-T-14	0.158816878	0.124569776	0.0777858283
Product	2.562139e-20	8.539343e-22	5.589708e-23
JU-C-2	0.71339980	ND	ND
JU-C-3	0.4093030	ND	ND
JU-C-4	0.9384669	ND	ND
JU-C-5	0.5475865	ND	ND
JU-C-6	0.7882016	ND	ND
JU-C-7	0.7383550	ND	ND
JU-C-8	0.6470016	ND	ND
Product	0.05650108	ND	ND

6. Methods

Ecdysone Injection Setup

All larval injections were done using the Nanoject Auto-Nanoliter Injector (Drummond Scientific Company, Broomall, PA). Injection needles were pulled from 3 ½" glass capillaries (#3-000-203-G/X, Drummond Scientific) using the Model 700C DKI Vertical Pipette Puller (David Kopf Instruments, Tujunga, CA). The settings for DKI Vertical Pipette Puller were as follows: heater, 45; solenoid, 50. The tip of the pulled needle was tapered under a dissecting scope using forceps. Needles were backfilled with mineral oil using a syringe, then affixed to the injector, then filled with the injection solution.

Ecdysone Injections and Salivary Gland DNA Extraction

All ecdysone injections were done on Early-Eyepost-stage, fourth instar female larvae from the Holo 2 stock. To prepare a single sample of salivary gland DNA, 10-15 larvae were anaesthetized with CO_2 and injected with 32 nanoliters of a solution consisting of 1 mg/ml 20-hydroxyecdysone, 50% ethanol, and blue dextran as a tracer, following the conditions by Foulk et al. (2006). Control larvae were injected with 32 nanoliters of 50% ethanol containing blue dextran. Injected larvae were incubated in a 21°C incubator for 24 hours on a 2.2 % Bacto agar plates (Becton, Dickinson and Company, Sparks, MD). For ecdysone-injected larvae, five larvae with a prematurely-induced Edge-Eye stage phenotype were selected from the plate, and the remaining larvae were discarded. We reasoned that larvae that did not prematurely progress to an Edge-Eye phenotype were not

successfully injected with ecdysone (for example, the needle passed straight through the larva). Salivary glands from each of the five larvae were dissected in Robert's CR buffer (Robert, 1971). Salivary gland DNA was extracted using DNAzol reagent as per the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA) and eluted in UltraPure DNase/RNase-Free Distilled Water (Thermo Fisher Scientific). Salivary gland DNA from control larvae was extracted using the same procedure. Purity and concentration was assessed using the NanoDrop fluorospectrometer (Thermo Fisher Scientific) and the Qubit Fluorometer using the Qubit dsDNA High Sensitivity Assay Kit (Thermo Fisher Scientific). Altogether, three DNA samples, each extracted from five pairs of salivary glands, were prepared for both the ecdysone and control-injected larvae (six samples total). The procedure is the same for 48 hour and 16 hour injections except for the length of the incubation.

Real-time Quantitative PCR

Calibrator genomic DNA for real-time PCR of salivary gland genomic DNA was extracted from female Holo 2 adult flies using DNAzol Reagent following the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA), and eluted in UltraPure DNase/RNase-Free Distilled Water (Thermo Fisher Scientific). Real-time PCR was performed using 14 target primer pairs flanking a region within candidate amplicon sequences determined from regions of high copy number from the genome assembly, and 8 control primer pairs flanking a non-amplified region. Each of these internal control sites is located on a contig also containing a

candidate amplicon sequence. A 1X reaction mix contained 7.5 µl 2X Power SYBR Green PCR Master Mix (Thermo Fisher Scientific), 2.5 µl UltraPure DNase/RNase-Free Distilled Water (Thermo Fisher Scientific), 1.5 µl of 1 µM forward primer, 1.5 µl of 1 µM reverse primer, and 2 µl of 50 pg/µl sample DNA. Real-time PCR was performed on an ABI 7300 Real-time PCR System for 40 cycles, followed by a dissociation stage (Applied Biosystems, Thermo Fisher Scientific). Three biological replicates for both ecdysone and control-injection salivary gland genomic DNA (DNA extraction of these biological replicates is explained above) were analyzed by real-time PCR. For each biological replicate, three technical replicates of a sample were included on a plate for a particular primer pair. Fold enrichment was calculated using the ΔΔCt method in RStudio, following established protocols (Livak & Schmittgen 2001, Schmittgen & Livak 2008, Weaver et al. 2010). Briefly, for both the sample and calibrator genomic DNA, ΔCt was calculated as $Ct_{amplicon}$ - $Ct_{internal\_control}$. Next ΔΔCt was calculated as ΔCt_{sample} - $\Delta Ct_{calibrator}$. Finally, fold amplification was calculated as $2^{-\Delta\Delta Ct}$. Ct values were excluded if they did not meet a 2% coefficient of variation cutoff, or if a well showed an abnormal dissociation curve.

Real-time Quantitative PCR Primers

o-JU-target1-fwd-1 5'-CCACTGTCACATCATCATCGCC-3'
o-JU-target1-rev-1 5'-GCGACGTTGCCTGTCAATCC-3'
o-JU-target2-fwd-1 5'-TATATGAGGCGAGGCCGAGG-3'
o-JU-target2-rev-1 5'-CGTTTCCGGTTCTCCCTCAC-3'
o-JU-target3-fwd-1 5'-CTCCGCTACGCTCAACAACA-3'
o-JU-target3-rev-1 5'-CATGGCCATCACCGAAGACA-3'
o-JU-target4-fwd-1 5'-GACCACTAACGTAAGCCGTGAA-3'
o-JU-target4-rev-1 5'-AAATTCCCAAAGGTGGATGCGA-3'

o-JU-target5-fwd-1 5’-ATCGCTTGATCGCTGGCAAA-3’
o-JU-target5-rev-1 5’-TTACCGTCCACTACACCCACA-3’
o-JU-target6-fwd-1 5’-TCGGCGCATAATGAACCTGAAA-3’
o-JU-target6-rev-1 5’-GCCCTTGAACAGAACCTTCCC-3’
o-JU-target7-fwd-1 5’-GGCAACGAGCCGATAAGGTC-3’
o-JU-target7-rev-1 5’-TATGTCAGCGCTGGTTTGGG-3’
o-JU-target8-fwd-1 5’-ATTCCGTGCCTCCCGATCTT-3’
o-JU-target8-rev-1 5’-AGTGTATGAAGACACTGCGCC-3’
o-JU-target9-fwd-1 5’-CGCCCACGGACAATCCTTAC-3’
o-JU-target9-rev-1 5’-CTCGAAAGAGCGTTGCCAGA-3’
o-JU-target10-fwd-1 5’-GCTTATTCAGCGAATGGAGTGGA-3’
o-JU-target10-rev-1 5’-GTAGTGTACGGTGGAAACGGG-3’
o-JU-target11-fwd-1 5’-CCAAACGAGGAGACGCCATT-3’
o-JU-target11-rev-1 5’-TATGCCTGGCGAGGTCTTGA-3’
o-JU-target12-fwd-1 5’-CGGCTCAGCGGTTCTACCTA-3’
o-JU-target12-rev-1 5’-CGCGACGTTGCGTCTGAAA-3’
o-JU-target13-fwd-1 5’-GGCATAGACCAACATGGATCA-3’
o-JU-target13-rev-1 5’-TCGACACTTTTTGCAATGCTC-3’
o-JU-target14-fwd-1 5’-GCTACAGCTGGTGGTCGAGA-3’
o-JU-target14-rev-1 5’-CGAACTGGGCCTCATGCTTT-3’
o-JU-control1-fwd-1 5’-TGCGAGGTATGATTTACCGTCTT-3’
o-JU-control1-rev-1 5’-TAGTGCGATGGCGAATTGATGT-3’
o-JU-control2-fwd-1 5’-CGGAAGGACGGCTACGAGAA-3’
o-JU-control2-rev-1 5’-GGAATGTCGTCCTGCCGTAAA-3’
o-JU-control3-fwd-1 5’-ATACCCAGACCCAGGTGGTAGA-3’
o-JU-control3-rev-1 5’-AAGTGCTGTGGGATAGCGAAGT-3’
o-JU-control4-fwd-1 5’-GATGCCATTCGCCGATAGTGTT-3’
o-JU-control4-rev-1 5’-TTCACTCACGCGATTCTCACAC-3’
o-JU-control5-fwd-1 5’-GGGCAACCAACGAAAAGTGG-3’
o-JU-control5-rev-1 5’-TCGGATTCCGGCTCCATACA-3’
o-JU-control6-fwd-1 5’-TCCGAGCCATAATCCTCACCTT-3’
o-JU-control6-rev-1 5’-TCGCACGCTAACGAAGGTATCA-3’
o-JU-control7-fwd-1 5’-ATTGTGGCGCAAGTCGAATCA-3’
o-JU-control7-rev-1 5’-ATGACATCGCTCATGTCGGG-3’
o-JU-control8-fwd-1 5’-GTTTCATCGGGAGGAACGGG-3’
o-JU-control8-rev-1 5’-TGGGACACGACAACATCAACTAC-3’

Sciara Larval Salivary Gland RNA Extraction

For the Northern blot, total RNA from *Sciara* larval salivary glands was extracted and size separated into small and large RNA fractions using the mirVana miRNA Isolation Kit (AM1561, AM9720, Ambion, Thermo Fisher Scientific), following

manufacturer's instructions except for two adjustments. The first, recommended by Dr. Jason Wood, is an on-column DNase digestion at step F.II.5, using the Qiagen RNase-Free DNase Set (#79254, Qiagen, Valencia, CA) following manufacturer's instructions for on-column DNase digestion. The second adjustment, to increase the size selection boundary, was to change the final ethanol concentration of the RNA sample from 25% to 18% at step F.II.1. RNA was eluted in UltraPure DNase/RNase-Free Distilled Water (Thermo Fisher Scientific). Concentration and purity was assessed using the NanoDrop fluorospectrometer (Thermo Fisher Scientific), and by running on 15% Polyacrylamide Gel Electrophoresis (7.2 g Urea, 3 ml 5X TBE, 5.6 ml 40% Acrylamide (acryl:bis acryl 19:1), UltraPure Water up to 15 ml, 75 ul 10% Ammonium Persulfate, 15 ul TEMED).

5.8S rDNA and 1kb II/9A ORI Cloning

NCBI Primer-BLAST was used to design primers flanking a region within the 5.8S RNA sequence in *Sciara*, obtained from Jordan et al. (1980). The following primers were used to amplify an 88 bp PCR product from adult *Sciara* genomic DNA.

5.8S Forward 5'-TGTGGGTCGATGAAGAACGC-3'

5.8S Reverse 5'-CCGCAATATGCGTTCAAAATGTCA-3'

The 1kb II/9A ORI fragment was provided by Dr. Yutaka Yamamoto in a pBluescript II SK(-) plasmid vector. The 1kb II/9A ORI insert was PCR amplified from the vector using the following primers:

Ori 1kb fwd 5’-GGATCCCCCCTGGGAGAGAAAGATATG-3’

Ori 1kb rev 5’ -CTTCGAAGAATTTTTTTGAAAGTGGTTTGGCTCTTTG-3’

Following PCR amplification of the 88bp 5.8S and 1kb II/9A sequences, the PCR product was run on 2% and 1% agarose gels respectively, extracted using the QIAquick Gel Extraction Kit (#28704, Qiagen), and eluted in UltraPure DNase/RNase-Free Distilled Water (Thermo Fisher Scientific)

The purified PCR products were cloned into pCR-Blunt II-TOPO plasmid vectors using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen, Thermo Fisher Scientific), electroporated into One-Shot Stbl3 *E. coli* (Invitrogen, Thermo Fisher Scientific) and cultured in 30 ug/ul kanamycin-containing LB medium for 24 hours following the instructions of the Zero Blunt TOPO PCR Cloning Kit. After culture, plasmids were isolated using the QIAprep Spin Miniprep Kit (#27106, Qiagen) and eluted in UltraPure DNase/RNase-Free Distilled Water (Thermo Fisher Scientific). Presence of the insert in the isolated plasmid was verified by restriction analysis with EcoRI-HF (R3101, New England Biolabs Inc., Ipswich, MA), and by sequencing using Genewiz with M13 primers against the M13 priming sites flanking the insertion site in the pCR-Blunt II-TOPO vector.

Northern Blot

The completed Northern blot procedure has not yet been done except for the initial small RNA extraction from larval salivary glands and gel electrophoresis of RNA

using a 15% acrylamide/urea gel, both of which have been detailed in sections of the Methods above.

Optimization results from subsequent steps, including electrotransfer to a nylon membrane, cross-linking of RNA to the nylon membrane, probe labelling, pre-hybridization, hybridization, washing, and exposure, will be included as an amendment to this thesis once they are completed.

7. References

1. Aladjem MI and Fanning E (2004). The replicon revisited: an old model learns new tricks in metazoan chromosomes. EMBO Rep. 5: 686-691.
2. Aladjem MI (2007). Replication in context: dynamic regulation of DNA replication patterns in metazoans. Nature Rev. Genet. 8: 588-600.
3. Aladjem MI & Redon CE. (2017) Order from clutter: selective interactions at mammalian replication origins. Nature Reviews Genetics 18:101-116.
4. Alexander JL, Inmaculada M, & Orr-Weaver TL. (2015). Replication fork progression during re-replication requires the DNA damage checkpoint and double strand break repair. Current Biology 25:1654-1660.
5. Bell, SP and Dutta A (2002). DNA replication in eukaryotic cells. Ann. Rev. Biochem. 71: 333-374.
6. Bielinsky A-K and Gerbi SA (2001). Where it all starts: eukaryotic origins of DNA replication. J. Cell Science 114: 643-651. PMID: 11171369.
7. Bienz-Tadmor B, Smith HS and Gerbi SA (1991). The promoter of DNA puff genes II/9-1 of Sciara coprophila is inducible by ecdysone in late prepupal salivary glands of Drosophila melanogaster. Cell Regul. (Mol. Biol. Cell) 2: 875-888. PMID: 1809395.
8. Blow JJ and Gillespie PJ. (2008). Replication licensing and cancer--a fatal entanglement? Nat Rev Cancer 8: 799-806.
9. Brewer BJ, Payen C, Raghuraman MK and Dunham MJ. (2011). Origin-dependent inverted-repeat amplification: a replication-based model for generating palindromic amplicons. PLoS Genet. 7:e1002016.
10. Candido-Silva JA, Machado MCR, Hartfelder KH, de Almeida JC, Paçó-Larson, ML, & Monesi N. (2015) Amplification and expression of a salivary gland DNA puff gene in the prothoracic gland of *Bradysia hygida* (Diptera: Sciaridae). Journal of Insect Physiology 74:30-37.
11. Chen S and Bell SP (2011). CDK prevents Mcm2-7 helicase loading by inhibiting Cdt1 interaction with Orc6. Genes Dev. 125: 363-372.
12. Claycomb JM, MacAlpine DM, Evans JG, Bell SP and Orr-Weaver TL (2002). Visualization of replication initiation and elongation in Drosophila. J Cell Biol. 159: 225-236.
13. Claycomb JM and Orr-Weaver TL (2005). Developmental gene amplification: insights into DNA replication and gene expression. Trends Genet. 21: 149-162.
14. Collart C, Christov CP, Smith JC, & Krude T. (2011). The midblastula transition defines the onset of Y RNA-dependent DNA replication in Xenopus laevis. Mol. Cell. Biol. 31(18):3857-3870.
15. Crouse HV (1968). The role of ecdysone in DNA puff formation and DNA synthesis in the polytene chromosomes of Sciara coprophila. Proc. Nat. Acad. Sci. 61: 971-978.
16. Crouse HV and Keyl H-G (1968). Extra replications in the "DNA puffs" of Sciara coprophila. Chromosoma 25: 357-364.
17. Delidakis C and Kafatos FC (1989). Amplification enhancers and replication origins in the autosomal chorion gene cluster of Drosophila. EMBO J. 8: 891-901.

18. DePamphilis ML, Blow JJ, Ghosh S, Saha T, Noguchi K and Vassilev A (2006). Regulating the licensing of DNA replication origins in metazoa. Curr Opin Cell Biol. 18:231-239.
19. DePamphilis ML and Bell SD (2010). Genome Duplication – concepts, mechanisms, evolution and disease (Garland Science, N.Y., N.Y.).
20. DiBartolomeis SM and Gerbi SA (1989). Molecular characterization of DNA puff II/9A genes in Sciara coprophila. J. Mol. Biol. 210: 531-540. PMID: 2614832.
21. Diffley JFX (2010). The many faces of redundancy in DNA replication control. Cold Spring Harbor Symp.Quant. Biol. 75: 135-142.
22. Dutta A and Bell SP (1997). Initiation of DNA replication in eukaryotic cells. Annu Rev Cell Dev Biol. 13: 293-332.
23. Farkas R & Mechler BM. (2000). The timing of drosophila salivary gland apoptosis displays an I(2)gl-dose response. Cell Death & Differentiation 7(1):89-101.
24. Foulk MS, Liang C, Wu N, Blitzblau H, Smith HS Alam D. Batra M, and Gerbi SA (2006). Ecdysone induces transcription and amplification in Sciara coprophila DNA puff II/9A. Dev. Biol. 299: 151-163. PMID: 16938289.
25. Foulk MS, Waggener JM, Johnson JM, Yamamoto Y, Liew GM, Urnov FD, Young Y, Lee G, Smith HS and Gerbi SA (2013). Isolation and characterization of the ecdysone receptor and its heterodimeric partner ultraspiracle through development in Sciara coprophila. Chromosoma 122: 103-119. PMID: 23321980; PMCID: PMC3964788.
26. Gabrusewycz-Garcia N (1964). Cytological and autoradiographic studies in Sciara coprophila salivary gland chromosomes. Chromosoma 15: 312-344.
27. Gabrusewycz-Garcia N (1971). Studies in polytene chromosomes of Sciarids. I. The salivary gland chromosomes of Sciara (Lycoriella) pauciseta (II), Felt. Chromosoma 33: 421-435.
28. Ge XQ & Lin H. (2014). Noncoding RNAs in the regulation of DNA replication. Trends in Biochemical Sciences, Cell Press 39(8):341-343.
29. Gerbi SA, Liang C, Wu N, DiBartolomeis SM, Bienz Tadmor B, Smith HS and Urnov FD (1993). DNA amplification in DNA puff II/9A of Sciara coprophila. Cold Spring Harbor Symp. Quant. Biol. 58: 487-494. PMID: 7956063.
30. Gerbi SA, Strezoska Z, & Waggener JM. Initiation of DNA replication in multicellular eukaryotes. (2003). Journal of Structural Biology 140:17-30.
31. Gopalakrishnan V, Simancek P, Houchens C, Snaith HA, Frattini MG, Sazer S, and Kelly TJ. (2001). Redundant control of rereplication in fission yeast. Proc. Nat. Acad. Sci. 98: 13114-13119.
32. Green BM, Finn KJ and Li JJ (2010). Loss of DNA replication control is a potent inducer of gene amplification. Science 329: 943-946. See Commentary by S.Kaochar, A.L.Paek and T.Weinert (2010). Replication error amplified. Science 329: 910-911.
33. Hanahan D & Weinberg RA (2011). Hallmarks of cancer: The next generation. Cell 144:646-674.

34. Heck MM and Spradling AC (1990). Multiple replication origins are used during Drosophila chorion gene amplification. J Cell Biol. 110:903-914.
35. Heller RC, Kang S, Lam WM, Chen S, Chan CS and Bell SP (2011). Eukaryotic origin-dependent DNA replication in vitro reveals sequential action of DDK and S-CDK kinases. Cell 146: 80-91.
36. Jordan BR, Latil-Damotte M, & Jourdan R. (1980). Coding and spacer sequences in the 5.8 S-2S region of *Sciara* coprophila ribosomal DNA. Nucleic Acids Res 8(16):3565-73.
37. Kiang L, Heichinger C, Watt S, Bahler J and Nurse P (2010). Specific replication origins promote DNA amplification in fission yeast. J. Cell Science 123: 3047-3051.
38. Kim JC and Orr-Weaver TL (2011). Analysis of a Drosophila amplicon in follicle cells highlights the diversity of metazoan replication origins. Proc Nat Acad Sci 108: 16681-16686.
39. Kim JC, Nordman J, Xie F, Kashevsky H, Eng T, Li S, MacAlpine DM and Orr-Weaver TL (2011). Integrative analysis of gene amplification in Drosophila follicle cells: parameters of origin activation and repression. Genes Dev 25:1384–1398.
40. Landis G & Tower J. (1999). The *Drosophila chiffon* gene is required for chorion gene amplification, and is related to the yeast Dbf4 regulator of DNA replication and cell cycle. *Development* 126: 4281-4293.
41. Landis G, Kelley R, Spradling AC, Tower J (1997). The k43 gene, required for chorion gene amplification and diploid cell chromosome replication, encodes the Drosophila homolog of yeast origin recognition complex subunit 2. Proc Natl Acad Sci 94: 3888-3892.
42. Laufer H, Rao B, & Nakase Y. (1967). Developmental studies of the dipteran salivary gland. IV. Changes in DNA content. Journal of Experimental Zoology 166(1):71-76.
43. Li A and Blow JJ *(*2005*).* Cdt1 downregulation by proteolysis and geminin inhibition prevents DNA re-replication in *Xenopus. EMBO J* 2: 395–404.
44. Liang C, Spitzer JD, Smith HS and Gerbi SA (1993). Replication initiates at a confined region during DNA amplification in Sciara DNA puff II/9A. Genes Dev 7: 1072-1084. PMID: 8504930.
45. Liang C and Gerbi SA (1994). Analysis of an origin of DNA amplification in Sciara coprophila using a novel three-dimensional gel method. Mol. Cell. Biol. 14: 1520-1529. PMID: 8289825.
46. Liew GM, M.S. Foulk and S.A. Gerbi. (2013). The ecdysone receptor (ScEcR-A) binds DNA puffs at the start of DNA amplification in Sciara coprophila.
47. Livak KJ & Schmittgen TD. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods 25:402-408.
48. Chromosome Res. 21(4):345-360. PMID: 23737076; PMCID: PMC3964806.
49. Mesner LD, Valsakumar V, Karnani N, Dutta A, Hamlin JL, & Bekiranov S. (2011). Bubble-chip analysis of human origin distributions demonstrates on

a genomic scale significant clustering zones and significant association with transcription. Genome Research 21: 377-389.
50. Miotto B, Ji Z, & Struhl, K. Selectivity of ORC binding sites and the relation to replication timing, fragile sites, and deletions in cancers. (2016). PNAS 133(33): 4810-4819.
51. Mohammad MM, Donti TR, Yakisich JS, Smith AG, & Kapler GM. (2007). The EMBO Journal 26(24):5048-5060.
52. Nguyen VQ, Co C and Li JJ (2001). Cyclin-dependent kinases prevent DNA re-replication through multiple mechanisms. Nature 411:1068-1073.
53. Nikolsky Y, Sviridov E, Yao J, Dosymbekov D, Ustyansky V, Kaznacheev V, Dezso Z, Mulvey L, Macconaill LE, Winckler W, Serebryiskaya T, Nikolskaya T, & Polyak K. (2008). Genome-wide functional synergy between amplified and mutated genes in human breast cancer. Cancer Res 68(22): 9532-9540.
54. Nordman J and Orr-Weaver TL (2012). Regulation of DNA replication during development. Development 139: 455-464.
55. Norseen J, Thomae A, Sridharan V, Aiyar A, Schepers A, & Lieberman PM. (2008). RNA-dependent recruitment of the origin recognition complex. The EMBO Journal. 27(22)3024-3035.
56. Poulson DF and Metz CW (1938). Studies on the structure of nucleolus-forming regions and related structures in the giant salivary gland chromosomes of Diptera. J. Morph. 63: 363-395.
57. Rasch EM (1970a). Two-wavelength cytophotometry of Sciara salivary gland chromosomes. In Introduction to Quantitative Cytochemistry (eds. G.L. Wied and G.F. Bahr), New York: Academic Press, vol. 2, pp. 335-355.
58. Rasch EM. (1970b). DNA cytophotometry of salivary gland nuclei and other tissue system in dipteran larvae. In: Wied, GL, Bahr. GF (Eds.), Introduction to Quantitative Cytochemistry. AcademicPress, New York, 335-355.
59. Remus D, Beall EL and Botchan MR (2004). DNA topology, not DNA sequence, is a critical determinant for Drosophila ORC-DNA binding. EMBO J. 23: 897-907.
60. Saxena S and Dutta A (2005). Geminin-Cdt1 balance is critical for genetic stability. Mutat Res. 569:111-121.
61. Schmittgen TD & Livak KJ (2008). Analyzing real-time PCR data by the comparative Ct method. Nature Protocols 3(6):1101-1108.
62. Schwed G, May N, Pechersky Y, & Calvi BR. (2002). *Drosophila* minichromosome maintenance 6 is required for chorion gene amplification and genomic replication. Mol Biol Cell 12:607-620.
63. Spradling AC. (1981). The organization and amplification of two chromosomal domains containing *Drosophila* chorion genes. Cell 27: 193-201.
64. Tower J (2004). Developmental gene amplification and origin regulation. Ann. Rev. Genet. 38: 273-304.
65. Weaver S, Dube S, Mir A, Qin J, Gang S, Ramakrishnan R, Jones RC, & Livak K. (2010). Taking qPCR to a higher level: Analysis of CNV reveals the

power of high throughput qPCR to enhance quantitative resolution. Methods 50:271-276.
66. Wohlschlegel JA, Dwyer BT, Dhar SK, Cvetic C, Walter JC and Dutta A. (2000). Inhibition of eukaryotic DNA replication by geminin binding to Cdt1. Science 290: 2309- 2312.
67. Wu N, Liang C, DiBartolomeis SM, Smith HS and Gerbi SA (1993). Developmental progression of DNA puffs in Sciara coprophila: amplification and transcription. Dev. Biol. 160: 73-84. PMID: 8224550.
68. Wu N, Liang C, DiBartolomeis SM, Smith HS and Gerbi SA (1993). Developmental progression of DNA puffs in Sciara coprophila: amplification and transcription. Dev. Biol. 160: 73-84. PMID: 8224550.
69. Yardimci H & Wlater JC. (2014). Prereplication-complex formation: a molecular double take? Nat. Struct. Mol. Biol. 21:20-25.