GENES AS MARKERS OF SEX FOR FORENSIC ENTOMOLOGY

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Genes as Markers of Sex for Forensic Entomology

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Calliphoridae is a large family of insects, and contains species *Lucilia sericata* (Diptera: Calliphoridae) (Meigen), *Cochliomyia macellaria* (Diptera: Calliphoridae) (Fabricius) and *Chrysomya rufifacies* (Diptera: Calliphoridae) (Macquart). These species are important medically and economically, and are commonly used in forensic investigations. In forensics, development data for species is used to predict time of colonization (TOC) estimates. However, there is sexual dimorphism in blow fly development and it is poorly understood. The difference in physical traits, as well as gene expression, may result in development disparities between sexes. For this reason, it is important to optimize a sex identification assay to aide in predicting more accurate TOC intervals for *L. sericata*, *C. macellaria* and *C. rufifacies*. Sex determination is an important assignment made in development. In the case of calliphorids, most undergo transformer (*tra*) splicing and resulting doublesex (*dsx*) splicing gives rise to downstream sex-specific characteristics. This may cause differing development in males and females leading to imprecise TOC estimates when not accounted for. Using known primer sets for *tra* and *dsx*, an assay for sex identification can be optimized. The newfound information on sex, in combination with published transcriptomes, can result in sex-specific interpretation of gene expression, yielding more accurate data sets for species.

DEDICATION

"Helping one person might not change the whole world, but it could change the world for one person"-Paul Shane Spear

I dedicate this work to my parents-Nancy and Gary Jonika, who have always believed in me and given me support through my pursuits in life. Your love and encouragement at all times has made it possible for me to be successful, and I hope those successes complete the dreams you have for me.

I would also like to thank all the hard working and dedicated teachers who never stopped believing in me and the potential that I had in life. Without that encouragement, I would not be where I am today.

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NOMENCLATURE

TRA	Transformer Protein
dsx	Doublesex
RT-PCR	Real-Time Polymerase Chain Reaction
PCR	Polymerase Chain Reaction
TOC	Time of Colonization
DNA	Deoxyribonucleic acid
sxl	Sex Lethal
tra	Transformer gene
XSL	X Signal Elements
tra^F	Female tra Transcript
fru	Fruitless
tra^M	Male tra Transcript
SXL	Sex Lethal Protein
RNA	Ribonucleic acid
DNase	Deoxyribonuclease
cDNA	Complementary DNA
PCR	Polymerase chain reaction
RT-PCR	Real-time polymerase chain reaction
mRNA	Messenger RNA
miRNA	Micro RNA
RNase	Ribonuclease

DNA	Deoxyribonucleic acid
EtOH	Ethanol
DNase I	Deoxyribonuclease I
EDTA	Ethylenediaminetetraacetic acid
RT	Reverse transcriptase
dNTP	Deoxynucleotide
UV	Ultraviolet

CHAPTER I

INTRODUCTION

Sex Determination in Animals

Sexual dimorphism, or the exhibition of differing characteristics between the two sexes, can evolve by two mechanisms - sexual selection and intraspecific niche divergence (Shine 1989). The importance of sexual dimorphism lies in the somatic cell differences that result in behavior, morphology and physiology (Mank 2009). Without sexual dimorphism, one sex would begin to lose or have bias, and natural selection favors equal expenditure of male and female progeny from parents (Fisher 1930, Slagsvold 1989). Traits such as pigmentation in *Drosophila* (Diptera: Drosophilidae) (Fallen), horn volume and body mass in bighorn sheep, facial adornments in primate species, and many other traits are attributed to sexual dimorphism (Dixson et al. 2005, Poissant et al. 2008, Williams et al. 2008). Recent genomic research has discovered evidence of many genes across the genome expressing sex-biased genome expression leading to male and female isoforms (Goldman and Arbeitman 2007), contributing to sex-specific phenotypes (Mank 2009).

Sex chromosomes are thought of as significant for their evolutionary importance in sex determination (Rice 1984, Ohno 2013, Mittwoch 2014). The determination of sex in animals is an important assignment that is made during development, and differs from species to species in animals; from the XY system to the ZW system or even environmental conditions during development (Bull 1983). The existence of two sexes allows for recombination and allele mixture within animals, and yields outcomes such as sexual reproduction and sexual dimorphism

(Archetti 2004, Crummett and Wayne 2009). Sexual dimorphism is of importance for this project as it gives rise to sex-specific differences within species.

Diptera: Calliphoridae

Calliphoridae, or blow flies, are an incredibly large and diverse family of insects that are typically found in tropical or warm environments (Whitworth 2006, Byrd and Castner 2009). They have characteristic blue, green or black metallic coloring on their thorax or abdomen (Whitworth 2006). This family is characteristically known as having bristles on their meron, well-developed calypters and plumose arista (Whitworth 2006). Flies and their immatures in this family are important forensically, medically and economically. This is due to their close association with decomposition (Sanford et al. 2014), their ability to cause myiasis and vector pathogens (Daeschlein et al. 2015), their usefulness in wound healing through maggot therapy (Peck and Kirkup 2015) and their infestation in livestock that can lead to fly strike (Hobson 1936, Hakimi and Yazdi 2002). Adult flies in this family occasionally pollinate and use nectar as their food source while larvae scavenge carrion and dung (Deyrup and Deyrup 2012). Calliphoridae is an incredibly diverse family that is comprised of 54 species in America, North of Mexico alone (Whitworth 2006). The three species of importance for this project are *Lucilia sericata* (Diptera: Calliphoridae) (Meigen), *Chrysomya rufifacies* (Diptera: Calliphoridae) (Macquart) and *Cochliomyia macellaria* (Diptera: Calliphoridae) (Fabricius).

Lucilia sericata

L. sericata, or the common green bottle fly, is commonly found worldwide from Southern Canada to Argentina and even Bermuda (Aubertin 1933, Woodley and Hilburn 1994).

It is often talked about in comparison with its sister species *Lucilia cuprina* (Diptera: Calliphoridae) (Wiedemann) (Aubertin 1933). It is characterized as having short, black setae, an orange basicosta, clear wings with light brown veins and white calypters (Whitworth 2006). *L. sericata* have forensic, veterinary and medical importance. In forensic investigations, the development can be used to calculate time of colonization (TOC) intervals (Tarone and Foran 2008). *L. sericata* are also commonly known to be agricultural pests for sheep by laying eggs and causing severe lesions and secondary bacterial infections underneath their wool (Aitken 2008). They also have medical importance in the treatment of bacterial infected wounds through a treatment known as maggot therapy (Horobin et al. 2003).

Cochliomyia macellaria

C. macellaria, or the secondary screwworm fly, is commonly found in warm, tropical environments (Byrd and Castner 2009). It is often talked about in comparison with its sister species *Cochliomyia homnivorax* (Diptera: Calliphoridae) (Coquerel), which has been eradicated from the United States (Wyss 2000). It is characterized as having orange gena, pale, white anterior spiracles, pale setulae on the frontal bristles and yellow basicosta (Whitworth 2006). *C. macellaria* are important forensically and medically. In forensic investigations, the development can be used to calculate TOC intervals (Wells and Greenberg 1992, Gupta and Setia 2004). They are also important medically as they were one of the first flies used in maggot therapy, though *L. sericata* is now the more common species used for treatment (Sherman et al. 2000, Bexfield et al. 2008). Though this species does consume flesh, it is often blamed for myiasitic attacks that are caused by its sister species, *C. homnivorax*, which is a huge economic pest in other parts of the world (Wells and Greenberg 1992, Gupta and Setia 2004).

Chrysomya rufifacies

C. rufifacies, or the hairy maggot blow fly, is widely distributed across the world, but prefers warmer environments (Dear 1985). It is often talked about in comparison with its sister species *Chrysomya megacephala* (Diptera: Calliphoridae) (Fabricius) (Byrd and Castner 2009) that is known for unique split eye phenotypes not common in most blow flies (Smith 2016). It is characterized as having a pale or white anterior thoracic spiracle, a greater ampulla with stiff erect setae, black abdominal tergites and setae on the meron (Whitworth 2006). *C. rufifacies* has forensic, economic and medical importance. In forensic investigations, the development can be used to calculate TOC intervals (Byrd and Butler 1997). The larvae are characteristically cannibalistic and maggots are predatory which can possibly affect TOC estimates (Baumgartner 1993). *C. rufifacies* have an economic effect through sheep strike in livestock and predation of *L. cuprina* and *L. sericata* maggots (Castner et al. 1995, Bram and George 2000). They also have been successfully used in maggot therapy and for predation of myiasis-causing flies (Baumgartner 1993, Bram and George 2000).

Importance of Calliphoridae in Forensic Investigations

Forensic entomology is the use of arthropods for legal and forensic purposes, and TOC is an important calculation within this discipline (Amendt et al. 2007, Catts 1992). TOC is an estimate of the time between death and colonization by arthropods of a corpse (Catts 1992). Natural decomposition processes such as rigor mortis and livor mortis can be used in time of death estimates but these processes only hold accurate for narrow windows of time (Campobasso et al. 2001, Bourel et al. 2003). However, insects both during the natural decomposition process and long after are a very powerful and accurate tool in estimating TOC (Amendt et al. 2011).

Insect activity resulting in decomposition is a process that can be measured continuously and provide accurate TOC estimates (Bourel et al. 2003). Behind these TOC estimates there is an assumption that calculating the age of insects developing on a body will allow for the calculation of a minimum post-mortem interval (minPMI) (Catts 1992). The focus of these intervals is on the family Calliphoridae as they are often the first colonizers of decomposing remains, so estimates usually involve this family for entomological evidence (Amendt et al. 2011). The development rate used in calculations for TOC is mainly based on temperature and therefore a few key pieces are needed in this process (Byrd and Castner 2009). A forensic entomologist must correctly identify a species, reconstruct the appropriate temperature and climate from the scene, and determine the rate of development for the specimens found on the corpse (Smith 1986, Archer 2004, Grassberger and Reiter 2001).

Though insect evaluation is the most accurate estimate for TOC, there remains some limitations in this methodology (Catts 1992). One such limitation is the ectothermic nature of insects and the heat that is given off by larval masses of blow flies as they are colonizing corpses (Amendt et al. 2011). Another is drugs and toxic substances that may have been present on the decomposing corpse and are ingested by the blow fly causing a change in the rate of development (Joseph et al. 2011). Lastly, an assumption of TOC estimations is that a minimum post-mortem interval is being calculated but in situations such as myiasis-the feeding of maggots on living flesh-this will not be the case (Catts 1992). Though these are limitations there is much literature that has been completed to overcome and mitigate these limitations to the greatest extent (Mathur and Agrawal 2011, Boehme et al. 2013, Buchan and Anderson 2001, Benecke and Wells 2001)). However, the greatest limitation and one that has not been greatly studied, is sexual dimorphism in the development of immature blow flies and a way to account for this

dimorphism in TOC estimates. In this project, I created an assay for three species of forensic importance- *L. sericata*, *C. macellaria*, and *C. rufifacies*-that will allow for sex determination in all specimens. Through the creation of these sex determination assays, a method was developed to account for differing development times in male and female blow flies in forensic applications. Though this work has previously been accomplished in some species for alternative applications (Li et al. 2013), this is the first work for sex determination assays for forensic science applications.

Sex-Determination Mechanisms in Calliphoridae

Model Fly: *Drosophila melanogaster* (Diptera: Drosophilidae) (Meigen)

Determination of male or female is an important decision in the development of any animal. The model fly, *D. melanogaster*, uses the activation of sex lethal (*Sxl*) master gene through high or low X signal element (XSL) expression levels (Cline 1993, Penalva and Sanchez 2003, Scott et al. 2014). High XSL expression levels activate *Sxl* and encode for females. The *Sxl* gene then regulates splicing for the female transformer (tra^F) gene, where the tra^F will code for a full-length transformer (TRA) protein (Inoue et al. 1990). The transformer 2 (TRA 2) protein then combines with tra^F to regulate the splicing of the transcript into either doublesex (*dsx*) or fruitless (*fru*) female (Hoshijima et al. 1991, Ryner and Baker 1991, Heinrichs et al. 1998). Low XSL expression levels direct male *tra* transcript (tra^M), lending rise to either *dsx* or *fru* male (Lynch and Maniatis 1996). As a model organism, most calliphorid species build off the *D. melanogaster* sex determination pathway that can be seen in Figure 1.1 below.

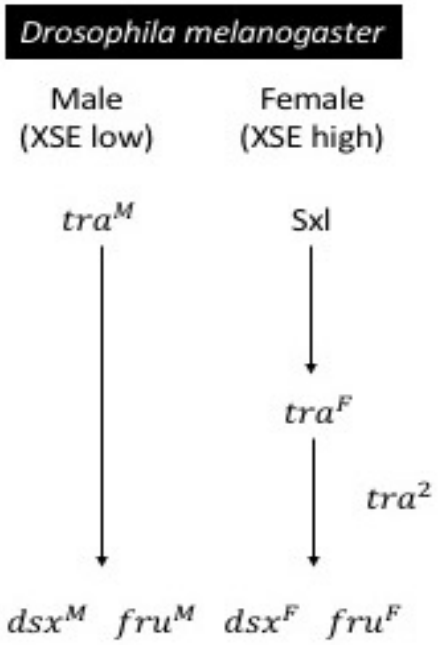

Figure 1.1. Sex determination mechanism of *D. melanogaster* (Modified from Scott et al. 2013).

Lucilia sericata

L. sericata tra genes have conserved regions and motifs when compared to its nearest relative, *L. cuprina*, which are important in the determination of sex splicing mechanism (McDonagh and Stevens 2011, DeBry et al. 2013). Unlike *D. melanogaster*, *tra* RNA splicing, not *Sxl,* is at the top of the hierarchy in females (Scott et al. 2014). tra^F and tra^M transcripts are determined through the first intron splicing site (Li et al. 2013). The presence of tra^M, inhibits *tra* RNA splicing while maternal TRA contributes to initiation of tra^F splicing (Scott et al. 2014). The mechanism after the point of *tra* splicing is like that of *D. melanogaster* with tra^F using tra-2 proteins to encode for either *dsx* or *fru* female and tra^M encoding for either *dsx* or *fru* male (Scott et al. 2014). A visual representation of the sex determination pathway of *L. sericata* can be seen in Figure 1.2 below.

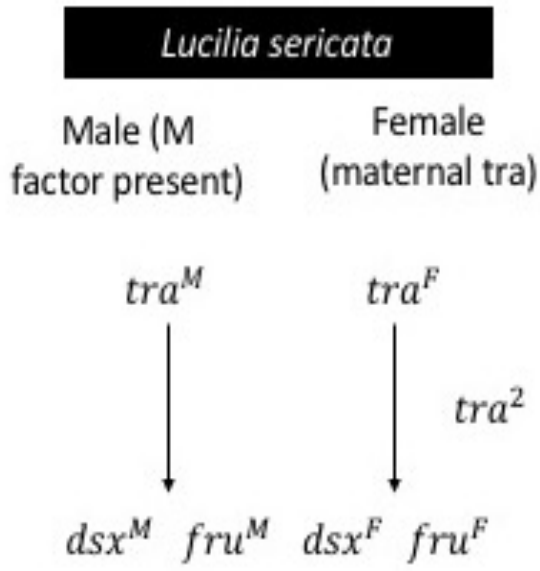

Figure 1.2. Sex determination mechanism of *L. sericata* (Modified from Scott et al. 2013).

Cochliomyia macellaria

C. macellaria tra genes also have conserved regions when compared with *L. cuprina* (Scott et al. 2014*)*. This conservation is useful for the determination and understanding of their sex splicing mechanism (Li et al. 2013). The *C. macellaria* mechanism is like that of *L. sericata* and *L. cuprina.* Through the first intron splicing sites, tra^F and tra^M are determined (Li et al. 2013). The tra^M products yield either *dsx* or *fru* males (Scott et al. 2014). The TRA-2 protein then combines with tra^F to either splice for *dsx* or *fru* females (Scott et al. 2014). Like *L. sericata*, the *tra* gene is auto regulated in female *C. macellaria (Li et al. 2013)*. A visual representation of the sex determination pathway of *C. macellaria* can be seen in Figure 1.3 below.

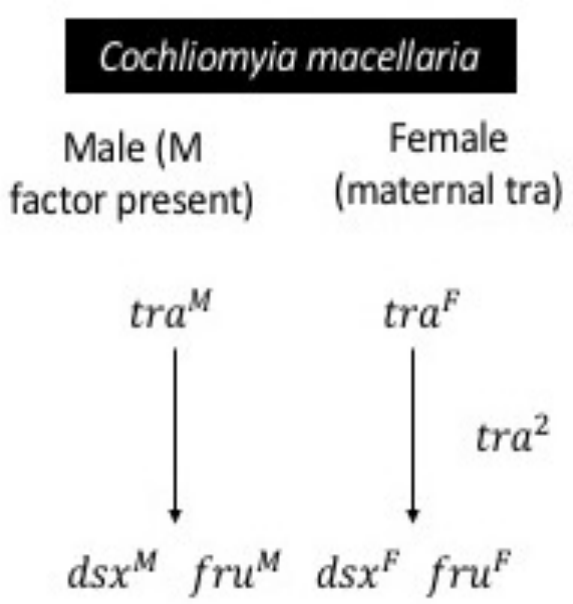

Figure 1.3. Sex determination mechanism of *C. macellaria* (Modified from Scott et al. 2013).

Chrysomya rufifacies

C. rufifacies differs from other calliphorid species in that it has monogenic sex determination, meaning females produce single-sex offspring broods (Roy and Siddons 1939, Wilton 1954). These broods are unique in that sex is determined independently of the mother's diet, and season or temperature experienced (Roy and Siddons 1939). Female producing, or thelygenic females, are heterozygous for a female determiner gene that is dominant, while male producing, or arrhenogenic females and males, are homozygous for the female determiner gene (Ullerich 1963). The female determiner gene has yet to be determined for *C. rufifacies*, but it is believed to be *dsx*. The sex lethal protein (SXL) is not important in the sex determination of *C. rufifacies* (Scott et al. 2014). A visual representation of the sex determination pathway of *C. rufifacies* can be seen in Figure 1.4 below.

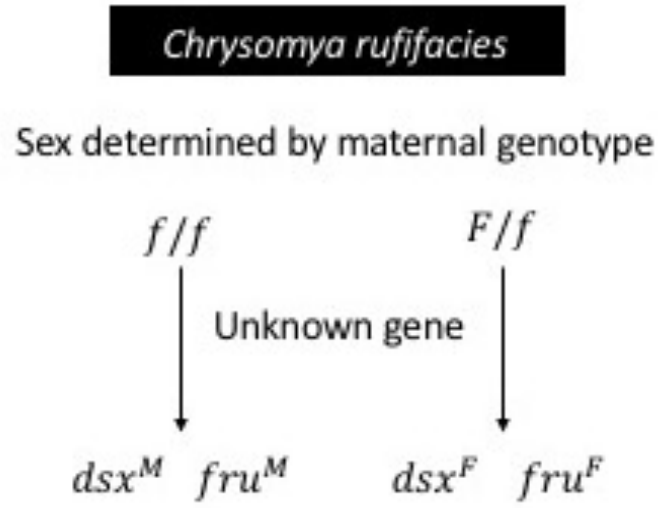

Figure 1.4. Sex determination mechanism of *C. rufifacies* (Modified from Scott et al. 2013).

Downstream of Doublesex and Fruitless

Doublesex splicing is often thought to give rise to morphological differences while fruitless splicing commonly gives rise to behavioral differences, though it also affects some morphological differences as well. Morphological characteristics that show different phenotypic traits based on sex in the doublesex pathway are foreleg sexcombs in *Drosophila*, regulation of pigmentation in *Drosophila* and other insects through genes such as bric-a-brac, and yolk protein expression (Burtis and Baker 1989, Wittkopp and Beldade 2009, Kopp 2011, Tarone et al. 2012). Fruitless splicing causes behavioral differences in sex such as courtship behavior in males and females and bisexual courtship caused by incorrect splicing, and morphological differences in the development of the muscle of Lawrence (Gailey et al. 1991, Ito et al. 1996, Demir and Dickson 2005).

Optimization of Sex Determination Assay

The objective for this study is to optimize a sex determination assay for the species: *L. sericata*, *C. macellaria*, and *C. rufifacies*. Optimization of a sex determination assay occurred through the use of previous methods outlined in Li et al. 2013.

Lucilia sericata

For *L. sericata*, gene expression data has been previously gathered for a holistic view of messenger RNA (mRNA) gene expression during larval and pupal stages. The genes chosen to analyze for expression data were gathered from the published transcriptome of *L. sericata* (Sze 2012). A sex determination assay would help to gain understanding in genes that are expressed sex specifically and if this affects sex-specific development of this species. If not using previous flies already analyzed for gene expression data, sex could be determined through genome sizing, as male and female *L. sericata* have differing, known genome sizes (Picard et al. 2012). However, the flies used for the gene expression study previously underwent RNA extraction so the ability to genome sizing for sex determination is not possible.

Using a published transcriptome, the splicing product of *L. sericata tra* can be targeted for optimization within the sex determination assay (Sze 2012). There is evidence of this being possible through methods used in Li et al. 2103, however, these methods were used for veterinary applications in male sterile release. Upon completion of this assay, this information can yield insight into understanding sex-specific gene expression and the effect this may have on development in *L. sericata*.

Cochliomyia macellaria

For *C. macellaria*, a sex determination assay needs to be created to better understand microRNA (miRNA) and protein expression in this species. Genes to analyze for gene expression were chosen from an in-house transcriptome of this species. The assay will allow us to choose only female flies to analyze for differences in sex-specific gene expression that give rise to differing developmental rates. The use of genome size is not possible for sex determination in this species as the male and female genome size are not distinguishable from one another (Picard et al. 2012).

Using an in-house transcriptome, the splicing product of *C. macellaria tra* can be targeted for optimization within the sex determination assay. There is evidence of this being possible through methods used in Li et al. 2103, however, these methods were used for veterinary applications in male sterile release. Upon completion of this assay, this information can yield insight into understanding sex-specific gene expression and the effect this may have on development in *C. macellaria*.

Chrysomya rufifacies

For *C. rufifacies*, a sex determination assay needs to be created to understand miRNA and protein expression in this species. Genes to analyze for gene expression will be chosen from the published transcriptome of this species (Sze et al. 2017). The assay will allow us to choose only female flies to analyze for differences in sex-specific gene expression that give rise to differing developmental rates. The use of genome size is not possible for sex determination in this species as the male and female genome size are not distinguishable from one another (Picard et al. 2012).

Using a published transcriptome, the splicing product of *C. rufifacies dsx* can be targeted for optimization within the sex determination assay (Sze et al. 2017). There is no known use of *C. rufifacies* splicing being used for sex determination. Upon completion of this assay, this information can yield insight into understanding sex-specific gene expression and the effect this may have on development in *C. rufifacies*.

CHAPTER II

METHODS

RNA Extraction and Quantification

TRI Reagent ® (Sigma-Aldrich Corp., St. Louis, Missouri, USA) was used to extract the RNA according to the instructions provided by the manufacturer. Each sample (i.e. a single larva or pupa) was placed into a 1.5 mL ribonuclease-free (RNAse-free) microfuge tube with 1 mL of cold TRI Reagent. The tissue was then ground with a sterile hand-held pestle. Next, 50 µL of ice-cold BAN reagent (Molecular Research Center, Inc., Cincinnati, Ohio, USA) were added to the tube and then the solution was vortexed for 15 seconds. The tubes were centrifuged for 15 minutes at 14,000 rpm at 4°C for isolation of RNA from deoxyribonucleic acid (DNA) and proteins. Approximately 500-600 µL of the top, clear, aqueous portion were pipetted out and placed into a new 1.5 mL RNAse-free microfuge tube along with 500 µL of ice-cold 100% isopropanol. The sample is mixed by inverting several times and then were kept on ice for 10 minutes to precipitate RNA. The tube was then centrifuged for 5 minutes at 14,000 rpm at 4 °C. The supernatant was removed and 1 mL of ice-cold 70% ethanol (EtOH) was mixed by pipetting to wash the pellet. The sample was centrifuged for 5 minutes at 14,000 rpm at 4°C then all EtOH was removed from the microfuge tube. The RNA pellet was dissolved in 100 µL of a mixture of 99 µL DNase/ RNase/ Nucleotide-free H_2O and 1 µL of SUPERase•IN™ (Invitrogen™, Life Technologies™ Inc., Grand Island, New York, USA). Quantification of the RNA was performed with a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific Inc.®, Wilmington, Deleware, USA). Samples that contained greater than 1,000 ng/µL of RNA were diluted with DNase/ RNase/ Nucleotide-free H_2O.

DNase I

Digestion of DNA and purification of the RNA before conversion to cDNA were performed using deoxyribonuclease I (DNase I) and Amplification Grade RNA (Invitrogen™). The concentration of the RNA was divided out of 100 to get the amount of RNA in µL. This amount was added into a 0.5 mL RNA-free tube on ice along with 2 µL DNase I. Enough DNase/ RNase/ Nucleotide-free H_2O was added to the reaction tube to bring the volume to 10 µL. The tube was incubated at room temperature for 15 minutes and then 1 µL of ethylenediaminetetraacetic acid (EDTA) was added to the solution. The samples were heated for 10 minutes at 65 ºC on a thermal cycler.

cDNA Conversion

The RNA was converted to cDNA with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Foster City, California, USA). Kit components were thawed on ice. A master mix was prepared with 2 µL 10X reverse transcriptase (RT) buffer, 0.8 uL25X deoxyribonucleotide (dNTP) Mix (100 mM), 2 µL 10X RT Random Primers, 1 µL MultiScribe Reverse Transcriptase and 4.2 µL DNase/ RNase/ Nucleotide-free H_2O for a total reaction volume of 10 µL. The 10 µL of master mix was added to 10 µL of the RNA sample in a 0.5 mL PCR strip tube on ice. The solution was mixed by vortexing to ensure mixture and then briefly spun down to eliminate air bubbles. The sample was converted to cDNA according to manufacturer protocols on a thermal cycler with the parameters seen in Table 2.1. After removal from the thermal cycler, the samples were kept at -20 °C.

Table 2.1. Thermal cycler parameters for cDNA conversion according to the manufacturer.

	Step 1	**Step 2**	**Step 3**	**Step 4**
Temperature (°C)	25	37	85	4
Time	10 minutes	120 minutes	5 minutes	∞

Primer Design

Primers for optimization within each species were either gathered from previous research or designed using published transcriptome data. All primers were used in a 1:10 dilution with DNase/ RNase/ Nucleotide-free H_2O. Detailed below is the primer design for each species of interest for this research.

Lucilia sericata

For *L. sericata*, there is a published de novo transcriptome assembly (Sze 2012). Li et al. 2013, utilized this known assembly under accession number JX315620, for targeting *tra* splicing within *L. sericata*. While these methods were for use in veterinary applications, these primers were useful in targeting the *tra* splicing for our sex determination assay for forensic applications. Li et al. 2013, used forward primer 5'-ATT TAA AAT TCA ACA ATC CAT ACC C-3' and reverse primer 5'-TCT AAA TTA TTA GTA TCA CGA GCA T-3'. Based on literature, an expected splicing product of 1,118 base pairs for males and 799 base pairs for females was expected. This primer set was then optimized through gradient polymerase chain reaction (PCR) for an assay that can determine sex in larvae and pupae as well as adults, for *L. sericata*.

Cochliomyia macellaria

For *C. macellaria*, there is an in-house de novo transcriptome assembly. Li et al. 2013, utilized this known assembly under accession number JX315619, for targeting *tra* splicing within *C. macellaria*. While these methods were for use in veterinary applications, these primers were useful in targeting the *tra* splicing for our sex determination assay for forensic applications. Li et al. 2013, used forward primer 5'-ATA CCA AGT GGT TCG GTG AAA AGA GGT C -3' and reverse primer 5'-GGT TTT AGT TTT ACC GCT TGT ATG GTG TTC -3'. Based on literature, an expected splicing product of 1,077 base pairs for males and 779 base pairs for females was expected. This primer set was then optimized through gradient PCR for an assay that can determine sex in larvae and pupae as well as adults, for *C. macellaria*.

Chrysomya rufifacies

For *C. rufifacies*, there is a published de novo transcriptome assembly (Sze et al. 2017). There is currently no literature that has attempted to use the *C. rufifacies* transcriptome for sex determination in any application. Using the known *dsx* node sequences for males (Appendix Figure 1.3) and females (Appendix Figure 1.2), and the common node sequence (Appendix A Figure 1.1, Primer-BLAST was used to locate ideal positioning for primers. The primers were restricted to a PCR product size of 70-1000 base pairs and a primer melting temperature between 57 °C and 63 °C. This primer set was then optimized through gradient PCR for an assay that can determine sex in larvae and pupae as well as adults, for *C. rufifacies*.

Gradient PCR

Lucilia sericata

For the *tra* PCR, a master mix of 10 µL H2O, 1.5 µL forward primer, 1.5 µL reverse primer and 15 µL master mix were added to a 1.5 mL RNAse-free microfuge tube. The 28 µL of master mix were added into a 0.5 mL PCR strip tube on ice along with 2 µL of cDNA for a total reaction volume of 30 µL. The samples were then placed onto the thermal cycler under the conditions in Table 2.2. The annealing temperature was varied for the 8 rows of samples within the thermal cycler. Samples were placed in the center of the thermal cycler with each row representing a different annealing temperature (Table 2.3). Results of the gradient PCR were viewed on a 1% agarose gel at 120V for ~1 hour and visualized under ultraviolet (UV) light.

Table 2.2. Gradient PCR conditions for *L. sericata*.

	Initial Incubation	**Denature**	**Anneal**	**Extend**	**Final Extension**	**Final Step**
Temperature (ºC)	95	95	Varied (Gradient)	72	72	4
Time	3 minutes	30 seconds	30 seconds	1 minute	4 minutes	∞

Table 2.3. Placement of samples on the thermal cycler with each row representing a different annealing temperature.

	1	2	3	4	5	6	7	8	9	10	11	12
A					♀ Neg	♂ Neg	Female	Male				
B					♀ Neg	♂ Neg	Female	Male				
C					♀ Neg	♂ Neg	Female	Male				
D					♀ Neg	♂ Neg	Female	Male				
E					♀ Neg	♂ Neg	Female	Male				
F					♀ Neg	♂ Neg	Female	Male				
G					♀ Neg	♂ Neg	Female	Male				
H					♀ Neg	♂ Neg	Female	Male				

Cochliomyia macellaria

For the TRA PCR, a master mix of 10 µL H2O, 1.5 µL forward primer, 1.5 µL reverse primer and 15 µL master mix were added to a 1.5 mL RNAse-free microfuge tube. The 28 µL of master mix were added into a 0.5 mL PCR strip tube on ice along with 2 µL of cDNA for a total reaction volume of 30 µL. The samples were then placed onto the thermal cycler under the conditions in Table 2.4. The annealing temperature was varied for the 8 rows of samples within the thermal cycler. Samples were placed in the center of the thermal cycler with each row representing a different annealing temperature (Table 2.5). Results of the Gradient PCR were viewed on a 1% agarose gel at 120V for ~1 hour and visualized under UV light.

Table 2.4. Gradient PCR conditions for *C. macellaria*.

	Initial Incubation	Denature	Anneal	Extend	Final Extension	Final Step
Temperature (ºC)	95	95	Varied (Gradient)	72	72	4
Time	3 minutes	30 seconds	30 seconds	1 minute	4 minutes	∞

Table 2.5. Placement of samples on the thermal cycler with each row representing a different annealing temperature.

	1	2	3	4	5	6	7	8	9	10	11	12
A					♀ Neg	♂ Neg	Female	Male				
B					♀ Neg	♂ Neg	Female	Male				
C					♀ Neg	♂ Neg	Female	Male				
D					♀ Neg	♂ Neg	Female	Male				
E					♀ Neg	♂ Neg	Female	Male				
F					♀ Neg	♂ Neg	Female	Male				
G					♀ Neg	♂ Neg	Female	Male				
H					♀ Neg	♂ Neg	Female	Male				

Chrysomya rufifacies

For the TRA PCR, a master mix of 10 µL H2O, 1.5 µL forward primer, 1.5 µL reverse primer and 15 µL master mix were added to a 1.5 mL RNAse-free microfuge tube. The 28 µL of master mix were added into a 0.5 mL PCR strip tube on ice along with 2 µL of cDNA for a total reaction volume of 30 µL. The samples were then placed onto the thermal cycler under the conditions in Table 2.6. The annealing temperature was varied for the 8 rows of samples within the thermal cycler. Samples were placed in the center of the thermal cycler with each row

representing a different annealing temperature (Table 2.7). Results of the Gradient PCR were viewed on a 1% agarose gel at 120V for ~1 hour and visualized under UV light.

Table 2.6. Gradient PCR conditions for *C. rufifacies*.

	Initial Incubation	**Denature**	**Anneal**	**Extend**	**Final Extension**	**Final Step**
Temperature (ºC)	95	95	Varied (Gradient)	72	72	4
Time	3 minutes	30 seconds	30 seconds	1 minute	4 minutes	∞

Table 2.7. Placement of samples on the thermal cycler with each row representing a different annealing temperature.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A					♀ Neg	♂ Neg	Female	Male				
B					♀ Neg	♂ Neg	Female	Male				
C					♀ Neg	♂ Neg	Female	Male				
D					♀ Neg	♂ Neg	Female	Male				
E					♀ Neg	♂ Neg	Female	Male				
F					♀ Neg	♂ Neg	Female	Male				
G					♀ Neg	♂ Neg	Female	Male				
H					♀ Neg	♂ Neg	Female	Male				

Real-Time PCR Analysis

For each plate, a no template control, a negative primer pair control and a positive control from the reverse transcriptase were ran. In each reaction, 5 µL of SSoFast™ EvaGreen® Supermix (Bio-Rad Laboratories, Inc.), 1 µL of forward primer (5'-ACA ATG TTA AGG AAC TCG AAG TTT TG-3'), 1 µL of reverse primer (5'-GGA GAC ACC GTG AGC GAT TT-3'), 1

µL of DNase/ RNase/ Nucleotide-free H2O, 2 µL of cDNA from the sample were loaded into a plate for a total reaction volume of 10 µL. The plate was run on RT-PCR with 40 cycles of denaturation at 94 ºC for 45 seconds and annealing/extension at 72 ºC for 60 seconds, followed by a 65 ºC-95 ºC melt curve at increments of 0.5 ºC. To ensure genomic DNA is not present in the samples, a reverse transcriptase check was also performed on all samples. A negative check with rp49 primers and the positive check with rp49 primers and a cDNA sample were analyzed for every sample in duplicate.

Error Rate Determination

To determine the error rate of the assays created, a double-blind testing of known sex determination was performed on 60 samples, 20 for each species. All samples underwent the same methodology performed in the optimization assays. However, rather than gradient PCR, standard PCR was performed using the ideal annealing temperature determined from optimizing the sex determination assay. In the tables below the placement of samples and standard PCR conditions for each species can be seen.

Lucilia sericata

In Table 2.8, the standard PCR conditions used for *L. sericata* can be seen along with the sample placement in Table 2.9.

Table 2.8. Standard PCR conditions for *L. sericata* sex determination samples.

	Initial Incubation	**Denature**	**Anneal**	**Extend**	**Final Extension**	**Final Step**
Temperature (ºC)	95	95	48.9	72	72	4
Time	3 minutes	30 seconds	30 seconds	1 minute	4 minutes	∞

Table 2.9. Placement of *L. sericata* sex determination samples on the thermal cycler with each row representing a different annealing temperature.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C		EL1	EL2	EL3	EL4	EL5	EL6	EL7	EL8	EL9	EL10	
D		EL1	EL2	EL3	EL4	EL5	EL6	EL7	EL8	EL9	EL10	
E	EL11	EL12	EL13	EL14	EL15	EL16	EL17	EL18	EL19	EL20	Neg	
F	EL11	EL12	EL13	EL14	EL15	EL16	EL17	EL18	EL19	EL20	Neg	
G												
H												

Cochliomyia macellaria

In Table 2.10, the standard PCR conditions used for *C. macellaria* can be seen along with the sample placement in Table 2.11.

Table 2.10. Standard PCR conditions for *C. macellaria* sex determination samples.

	Initial Incubation	Denature	Anneal	Extend	Final Extension	Final Step
Temperature (ºC)	95	95	57.7	72	72	4
Time	3 minutes	30 seconds	30 seconds	1 minute	4 minutes	∞

Table 2.11. Placement of *C. macellaria* sex determination samples on the thermal cycler with each row representing a different annealing temperature.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C		EM1	EM2	EM3	EM4	EM5	EM6	EM7	EM8	EM9	EM10	
D		EM1	EM2	EM3	EM4	EM5	EM6	EM7	EM8	EM9	EM10	
E	EM11	EM12	EM13	EM14	EM15	EM16	EM17	EM18	EM19	EM20	Neg	
F	EM11	EM12	EM13	EM14	EM15	EM16	EM17	EM18	EM19	EM20	Neg	
G												
H												

Chrysomya rufifacies

In Table 2.12, the standard PCR conditions used for *C. rufifacies* can be seen along with the sample placement in Table 2.13.

Table 2.12. Standard PCR conditions for *C. rufifacies* sex determination samples.

	Initial Incubation	**Denature**	**Anneal**	**Extend**	**Final Extension**	**Final Step**
Temperature (ºC)	95	95		72	72	4
Time	3 minutes	30 seconds	30 seconds	1 minute	4 minutes	∞

Table 2.13. Placement of *C. rufifacies* sex determination samples on the thermal cycler with each row representing a different annealing temperature.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A												
B												
C		ER1	ER2	ER3	ER4	ER5	ER6	ER7	ER8	ER9	ER10	
D		ER1	ER2	ER3	ER4	ER5	ER6	ER7	ER8	ER9	ER10	
E	ER11	ER12	ER13	ER14	ER15	ER16	ER17	ER18	ER19	ER20	Neg	
F	ER11	ER12	ER13	ER14	ER15	ER16	ER17	ER18	ER19	ER20	Neg	
G												
H												

CHAPTER III

LUCILIA SERICATA RESULTS

Gradient PCR

Gradient PCR for *L. sericata* was performed targeting the *transformer* gene *(tra)* within the differential splicing pathway. The selected forward primer has the sequence 5'-ATT TAA AAT TCA ACA ATC CAT ACC C-3' and the reverse primer has the sequence 5'-TCT AAA TTA TTA GTA TCA CGA GCA T-3'. For the first gradient PCR, annealing temperatures between 48 ºC and 62 ºC were used. In Table 3.1, the exact temperatures for each row of the gradient PCR can be seen. From this gradient PCR, bands at 48 ºC and 49 ºC were seen for female samples at approximately 200 base pairs and a band is present at 48 ºC for males at approximately 500 base pairs. These splicing products can be seen in Figure 3.1 below.

Table 3.1. Gradient PCR temperatures for *L. sericata* gel 1. Temperatures were varied within each row of the thermal cycler for one female and one male sample with cDNA and one female and one male negative each.

	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**
Temperature (ºC)	62.0	60.9	59.2	56.5	53.2	50.7	49.0	48.0

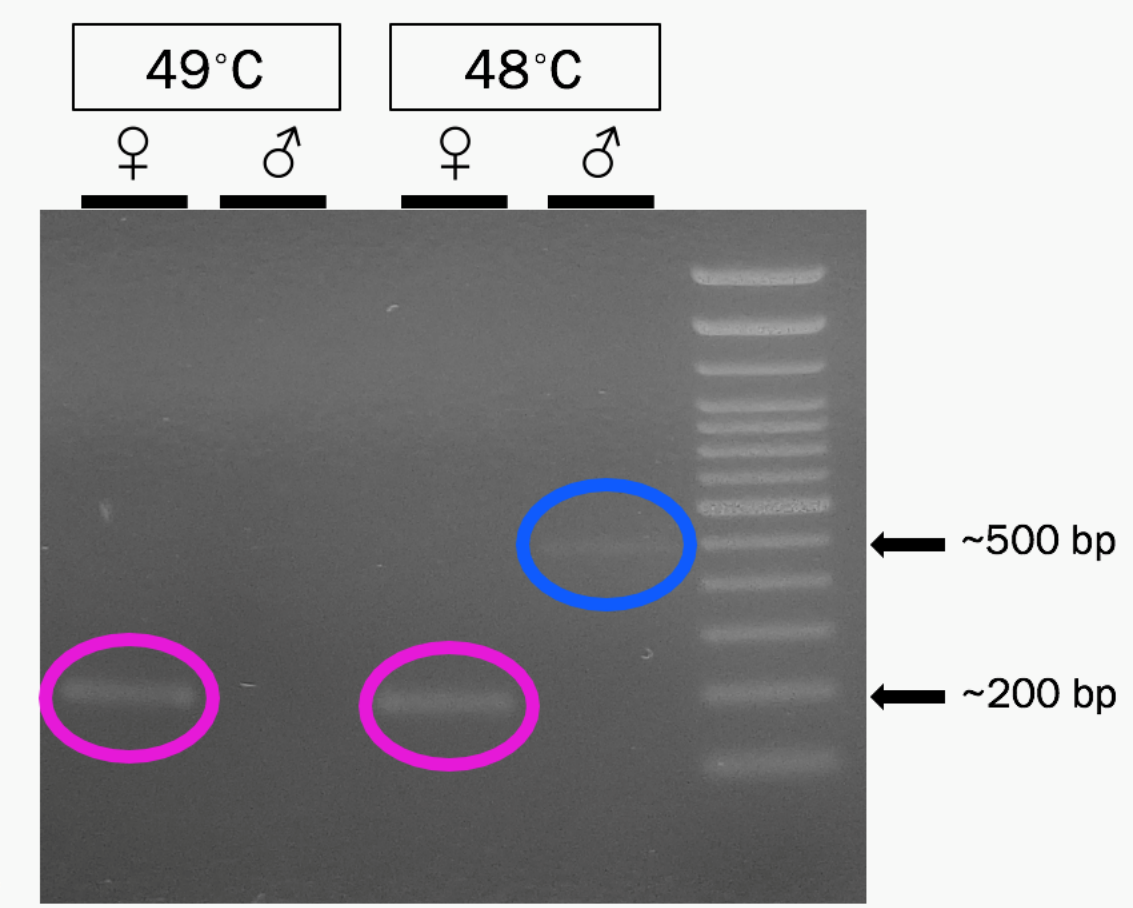

Figure 3.1. Agarose gel visualization of the gel product obtained from the first gradient PCR of species *L. sericata*. A female band can be seen at 48 ºC and 49 ºC at approximately 200 base pairs and a male band can be seen at 48 ºC at approximately 500 base pairs. No other distinct bands were present upon agarose gel visualization for other temperatures tested.

Upon visualization of the gradient PCR product, lower annealing temperatures were determined as the best for the selected primer pair. From here, another gradient PCR was run with annealing temperatures between 45 ºC and 49 ºC. In Table 3.2, the exact temperatures for each row of the gradient PCR can be seen. From this gradient PCR, bands at 45 ºC, 45.2 ºC, 45.7 ºC, 46.4 ºC, 47.3 ºC, 48.1 ºC, 48.6 ºC and 49 ºC were seen for females at approximately 200 base

pairs and no distinct male bands were present for any of the temperatures. These splicing products can be seen in Figure 3.2 below.

Table 3.2. Gradient PCR temperatures for *L. sericata* gel 2. Temperatures were varied within each row of the thermal cycler for one female and one male sample with cDNA and one female and one male negative each.

	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**
Temperature (ºC)	45.0	45.2	45.7	46.4	47.3	48.1	48.6	49.0

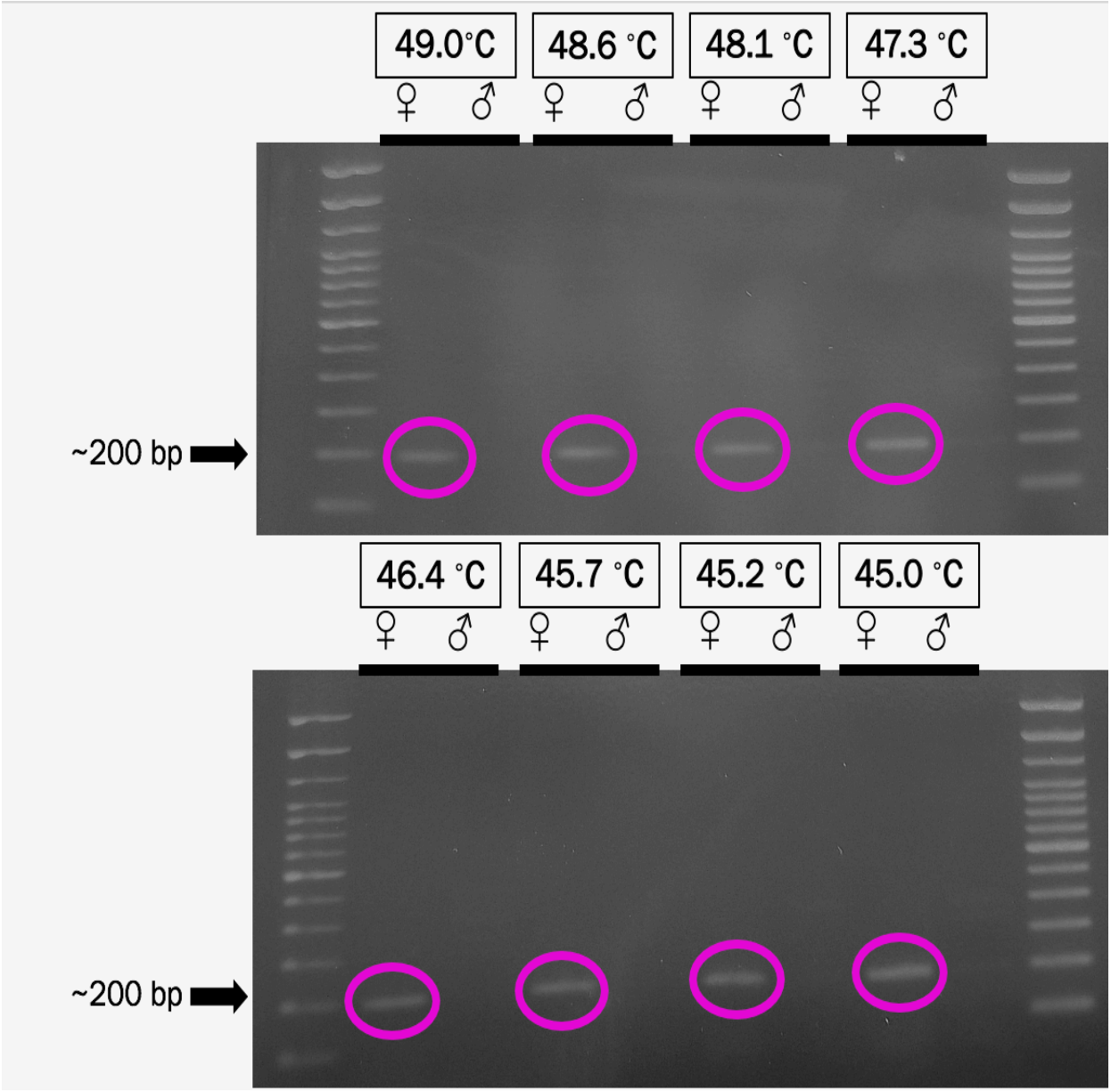

Figure 3.2. Agarose gel visualization of the gel product obtained from the second gradient PCR of species *L. sericata*. Female bands can be seen at 45 ºC, 45.2 ºC, 45.7 ºC, 46.4 ºC, 47.3 ºC, 48.1 ºC, 48.6 ºC and 49 ºC at approximately 200 base pairs and some faint male bands around 47.3 ºC and 48.1 ºC, but no distinct male bands are present at any temperature.

From the previous two gradient PCR products, we see that there appears to be faint bands for males potentially between 48 ºC and 49 ºC. From here, another gradient PCR was run with

annealing temperatures between 48 ºC and 49 ºC to see if we could get any better resolution for male bands at these temperatures. In Table 3.3, the exact temperatures for each row of the gradient PCR can be seen. From this gradient PCR, bands at 48.9 ºC and 49.0 ºC were seen for females at approximately 200 base pairs and no distinct male bands were present for any of the temperatures. These splicing products can be seen in Figure 3.3 below.

Table 3.3. Gradient PCR temperatures for *L. sericata* gel 3. Temperatures were varied within each row of the thermal cycler for one female and one male sample with cDNA and one female and one male negative each.

	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**
Temperature (ºC)	49.0	48.9	48.8	48.6	48.3	48.2	48.1	48.0

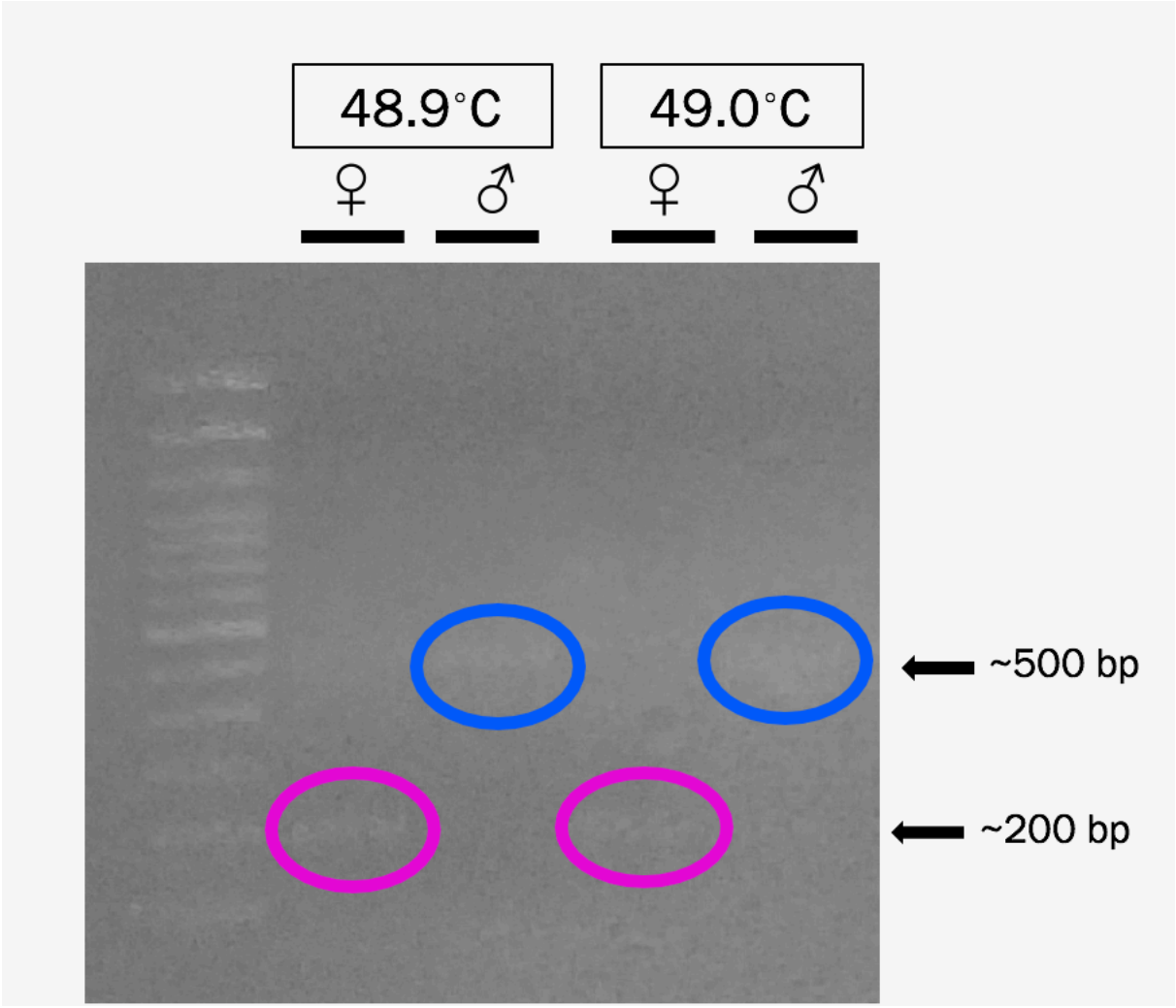

Figure 3.3. Agarose gel visualization of the gel product obtained from the third gradient PCR of species *L. sericata*. Faint female bands can be seen at 48.9 ºC and 49 ºC at approximately 200 base pairs and faint male bands can be seen at 48.9 ºC and 49.0 ºC. These bands are very faint and not as distinct as previous gradient PCR bands.

qPCR

All cDNA samples were analyzed on qPCR for statistical analysis and to complete a reverse transcriptase check (RT) to ensure no presence of genomic DNA. The housekeeper gene *rp49* was used to perform this check which has a known melt temperature of approximately 78.5

ºC (Faris 2017). All RT- samples were negative for the presence of genomic DNA and did not have the presence of a melt temperature while all RT+ samples had a melt temperature at approximately 78.5 ºC. The results for this can be seen in Tables 3.4 and 3.5 and the cycle fluorescence can be seen in Tables 3.6 and 3.7. Tables 3.4 and 3.6 show the first cDNA conversion for the first replicate of these samples. A second replicate of cDNA for these samples was needed after consumption of the first replicate and these cDNA values can be seen in Tables 3.5 and 3.7.

Table 3.4. qPCR results for melt temperatures for the RT+ and RT- samples for replicate 1 *L.sericata* samples.

Sample	Replicate cDNA sample	RT+ Melt Temperature (ºC)	RT- Melt Temperature (ºC)
L1	1	78.0	None
L2	1	78.5	None
L3	1	78.5	None
L4	1	78.5	None
L5	1	78.5	None
L6	1	78.5	None
L7	1	78.5	None
L8	1	78.5	None
L9	1	78.5	None
L10	1	78.5	None
L11	1	79.0	None
L12	1	79.0	None
L13	1	78.5	None
L14	1	78.5	None
L15	1	79.0	None
L16	1	78.5	None
L17	1	79.0	None
L18	1	79.0	None
L19	1	79.0	None
L20	1	78.5	None
L21	1	78.5	None

Table 5. qPCR results for melt temperatures for the RT+ and RT- samples for replicate 2 *L.sericata* samples.

Sample	Replicate cDNAsample	RT+ Melt Temperature (ºC)	RT- Melt Temperature (ºC)
L1	2	79.5	None
L2	2	79.5	None
L3	2	79.5	None
L4	2	78.5	None
L5	2	79.5	None
L6	2	79.5	None
L7	2	79.5	None
L8	2	79.5	None
L9	2	79.5	None
L10	2	79.5	None
L11	2	79.5	None
L12	2	79.5	None
L13	2	79.5	None
L14	2	79.0	None
L15	2	79.5	None
L16	2	79.5	None
L17	2	79.5	None
L18	2	79.5	None
L19	2	79.0	None
L20	2	79.0	None
L21	2	79.5	None

Table 6. qPCR results for cycle fluorescence for replicate 1 *L.sericata* samples.

Sample	Replicate cDNA sample	Cq Replicate 1	Cq Replicate 2	Cq Mean Replicate 1	Cq Mean Replicate 2	Cq Standard Deviation Replicate 1	Cq Standard Deviation Replicate 2
L1	1	29.94	28.97	29.46	29.46	0.682	0.682
L2	1	22.59	22.47	22.59	22.47	0.000	0.000
L3	1	26.62	25.31	25.97	25.97	0.924	0.924
L4	1	23.37	23.19	23.28	23.28	0.130	0.130
L5	1	19.73	19.57	19.65	19.65	0.108	0.108
L6	1	22.61	22.45	22.53	22.53	0.113	0.113
L7	1	20.28	20.37	20.32	20.32	0.068	0.068
L8	1	27.41	24.50	25.96	25.96	2.054	2.054
L9	1	30.37	22.13	26.25	26.25	5.829	5.829
L10	1	25.39	26.67	26.03	26.03	0.904	0.904
L11	1	20.87	20.96	20.91	20.91	0.068	0.068
L12	1	19.75	19.67	19.71	19.71	0.054	0.054
L13	1	N/A	38.84	N/A	38.84	N/A	N/A
L14	1	N/A	37.30	N/A	37.30	N/A	N/A
L15	1	20.33	20.25	20.29	20.29	0.056	0.056
L16	1	21.54	N/A	21.54	N/A	0.000	0.000
L17	1	27.13	26.71	26.92	26.92	0.297	0.297
L18	1	19.41	19.37	19.39	19.39	0.031	0.031
L19	1	N/A	N/A	N/A	N/A	N/A	N/A
L20	1	25.87	25.88	25.87	25.87	0.000	0.000
L21	1	26.39	35.24	30.81	30.81	6.257	6.257

Table 7. qPCR results for cycle fluorescence for replicate 2 *L.sericata* samples.

Sample	Replicate cDNA sample	Cq Replicate 1	Cq Replicate 2	Cq Mean Replicate 1	Cq Mean Replicate 2	Cq Standard Deviation Replicate 1	Cq Standard Deviation Replicate 2
L1	2	20.85	20.67	20.76	20.76	0.123	0.123
L2	2	22.31	22.47	22.39	22.39	0.110	0.110
L3	2	21.24	21.22	21.23	21.23	0.019	0.019
L4	2	30.96	29.62	30.29	30.29	0.952	0.952
L5	2	19.13	19.19	19.16	19.16	0.038	0.038
L6	2	21.43	21.38	21.41	21.41	0.033	0.033
L7	2	20.28	20.39	20.34	20.34	0.074	0.074
L8	2	20.79	20.67	20.73	20.73	0.085	0.085
L9	2	20.05	20.21	20.13	20.13	0.113	0.113
L10	2	19.93	19.68	19.81	19.81	0.177	0.177
L11	2	21.06	20.99	21.02	21.02	0.046	0.046
L12	2	19.14	19.10	19.12	19.12	0.038	0.038
L13	2	20.21	19.55	19.88	19.88	0.462	0.462
L14	2	19.09	21.51	20.30	20.30	1.716	1.716
L15	2	20.25	20.29	20.27	20.27	0.029	0.029
L16	2	18.60	18.58	18.59	18.59	0.017	0.017
L17	2	19.88	22.36	21.12	21.12	1.758	1.758
L18	2	19.69	19.54	19.62	19.62	0.109	0.109
L19	2	19.50	19.95	19.72	19.72	0.318	0.318
L20	2	22.13	23.86	23.00	23.00	1.220	1.220
L21	2	18.72	18.57	18.65	18.65	0.107	0.107

CHAPTER IV

COCHLIOMYIA MACELLARIA RESULTS

Gradient PCR

Gradient PCR for *C. macellaria* was performed targeting the *transformer* (*tra*) gene within the differential splicing pathway. The first primer pair, F 5'-CAT GCA ATT GTG CGT TCG GT-3' and R: 5'-CGT CTT CTT CTT GGC GGA CT-3', did not yield any results in either males or females. Another primer pair was selected targeting the transformer gene with a forward primer sequence 5'-ATA CCA AGT GGT TCG GTG AAA AGA GGT C-3' and reverse primer sequence 5'-GGT TTT AGT TTT ACC GCT TGT ATG GTG TTC-3'. For the first gradient PCR, annealing temperatures between 54 ºC and 64 ºC were used. In Table 4.1, the exact temperatures for each row of the gradient PCR can be seen. From this gradient PCR, bands at 60.1 ºC, 57.7 ºC, 55.9 ºC, 54.7 ºC, 54.0 ºC were seen for females at approximately 200 base pairs and bands at 62 ºC, 60.1 ºC, 57.7 ºC, 55.9 ºC, 54.7 ºC, 54.0 ºC were seen for males at approximately 500 and 350 base pairs. This can be seen in Figure 4.1 below.

Table 4.1. Gradient PCR temperatures for *C. macellaria* gel 1. Temperatures varied within each row of the thermal cycler for one female and one male sample with cDNA and one female and one male negative each.

	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**
Temperature (ºC)	54.0	54.7	55.9	57.7	60.1	62.0	63.2	64.0

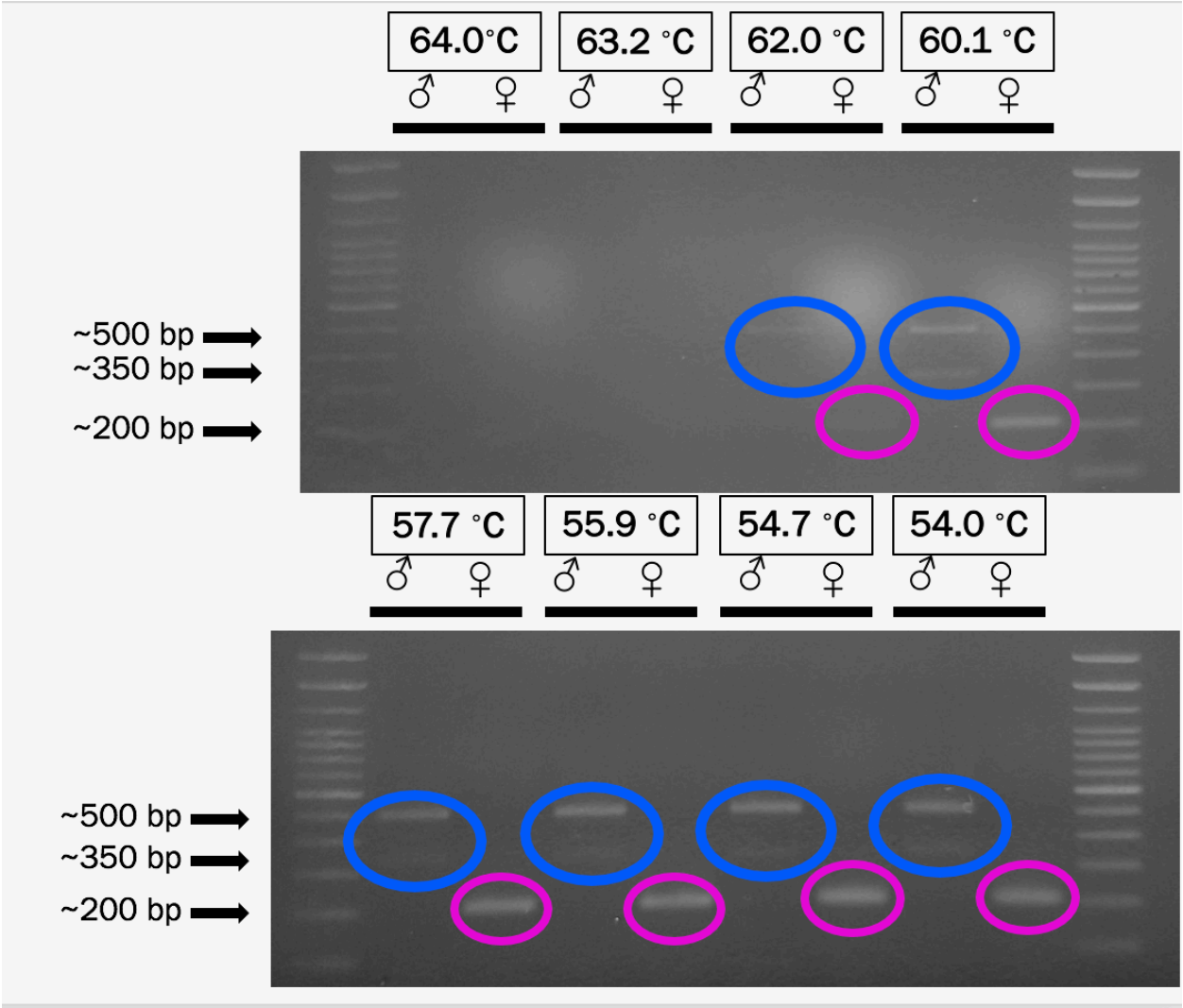

Figure 4.1. Agarose gel visualization of the gel product obtained from the first gradient PCR of species *C. macellaria*. Female bands can be seen at 54 ºC, 54.7 ºC, 55.9 ºC, 57.7 ºC, 60.1 ºC and 62.0 ºC at approximately 200 base pairs and male bands can be seen at 54 ºC, 54.7 ºC, 55.9 ºC, 57.7 ºC, 60.1 ºC and 62.0 ºC at approximately 350 and 500 base pairs.

qPCR

All cDNA samples were analyzed on qPCR for statistical analysis and to complete an RT check to ensure no presence of genomic DNA. The housekeeper gene *rp49* was used to perform this check which has a known melt temperature of approximately 78.5 ºC (Faris 2017, my dissertation). All RT- samples were negative for the presence of genomic DNA and did not have the presence of a melt temperature while all RT+ samples had a melt temperature at approximately 78.5 ºC. The results for this can be seen in Tables 4.2 and 4.3 and the cycle fluorescence can be seen in Tables 4.4 and 4.5. Tables 4.2 and 4.4 show the first cDNA conversion for the first replicate of these samples. A second replicate of cDNA for these samples was needed after consumption of the first replicate and these cDNA values can be seen in Tables 4.3 and 4.5.

Table 4.2. qPCR results for melt temperatures for the RT+ and RT- samples for replicate 1 *C. macellaria* samples.

Sample	Replicate	RT+ Melt Temperature (ºC)	RT- Melt Temperature (ºC)
M1	1	79.5	None
M2	1	79.5	None
M3	1	79.5	None
M4	1	79.5	None
M5	1	79.5	None
M6	1	79.5	None
M7	1	79.5	None
M8	1	79.5	None
M9	1	79.5	None
M10	1	79.5	None

Table 4.3. qPCR results for melt temperatures for the RT+ and RT- samples for replicate 2 *C. macellaria* samples.

Sample	Replicate	RT+ Melt Temperature (ºC)	RT- Melt Temperature (ºC)
M1	2	79.5	None
M2	2	79.5	None
M3	2	79.5	None
M4	2	79.5	None
M5	2	79.5	None
M6	2	80.0	None
M7	2	79.5	None
M8	2	80.0	None
M9	2	80.0	None
M10	2	79.5	None

Table 4.4. qPCR results for cycle fluorescence for replicate 1 *C. macellaria* samples.

Sample	Replicate	Cq Replicate 1	Cq Replicate 2	Cq Mean Replicate 1	Cq Mean Replicate 2	Cq Standard Deviation Replicate 1	Cq Standard Deviation Replicate 2
M1	1	22.93	22.12	22.52	22.52	0.578	0.578
M2	1	21.85	21.09	21.47	21.47	0.537	0.537
M3	1	21.62	22.04	21.83	21.83	0.296	0.296
M4	1	21.84	21.56	21.70	21.70	0.196	0.196
M5	1	22.47	21.75	22.11	22.11	0.514	0.514
M6	1	20.06	21.73	20.90	20.90	1.180	1.180
M7	1	19.67	21.24	20.46	20.46	1.111	1.111
M8	1	19.66	19.84	19.75	19.75	0.123	0.123
M9	1	19.64	19.63	19.63	19.63	0.012	0.012
M10	1	20.69	20.66	20.67	20.67	0.021	0.021

Table 4.5. qPCR results for cycle fluorescence for replicate 2 *C. macellaria* samples.

Sample	Replicate	Cq Replicate 1	Cq Replicate 2	Cq Mean Replicate 1	Cq Mean Replicate 2	Cq Standard Deviation Replicate 1	Cq Standard Deviation Replicate 2
M1	2	21.83	21.87	21.85	21.85	0.029	0.029
M2	2	21.07	21.29	21.18	21.18	0.157	0.157
M3	2	22.40	22.39	22.39	22.39	0.006	0.006
M4	2	21.38	20.81	21.10	21.10	0.403	0.403
M5	2	21.24	21.72	21.48	21.48	0.337	0.337
M6	2	20.31	20.78	20.54	20.54	0.335	0.335
M7	2	20.13	20.73	20.43	20.43	0.424	0.424
M8	2	20.30	20.02	20.16	20.16	0.194	0.194
M9	2	20.28	20.28	20.28	20.28	0	0
M10	2	20.73	20.75	20.74	20.74	0.011	0.011

CHAPTER V

CHRYSOMYA RUFIFACIES RESULTS

Gradient PCR

Gradient PCR for *C. rufifacies* was performed targeting the *doublesex* gene (*dsx*) within the differential splicing pathway. The selected forward primer has the sequence 5'-GCC ATG TTC CTG CTG CTC TA-3' and the reverse primer for females has the sequence 5'-ATT GTT GCT ACG TTG CTG CG-3'. No male reverse primer could be optimized for *C. rufifacies*. For the first gradient PCR, annealing temperatures between 54 ºC and 64 ºC were used. In table 5.1, the exact temperatures for each row of the gradient PCR can be seen. From this gradient PCR, bands at 64.0 ºC, 63.2 ºC, 62.0 ºC, 60.1 ºC, 57.7 ºC, 55.9 ºC, 54.7 ºC and 54.0 ºC were seen for females at approximately 700 base pairs and no distinct male bands were present. This can be seen in Figure 5.1 below.

Table 5.1. Gradient PCR temperatures for *C. rufifacies* gel 1. Temperatures were varied within each row of the thermal cycler for one female and one male sample with cDNA and one female and one male negative each.

	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**
Temperature (ºC)	54.0	54.7	55.9	57.7	60.1	62.0	63.2	64.0

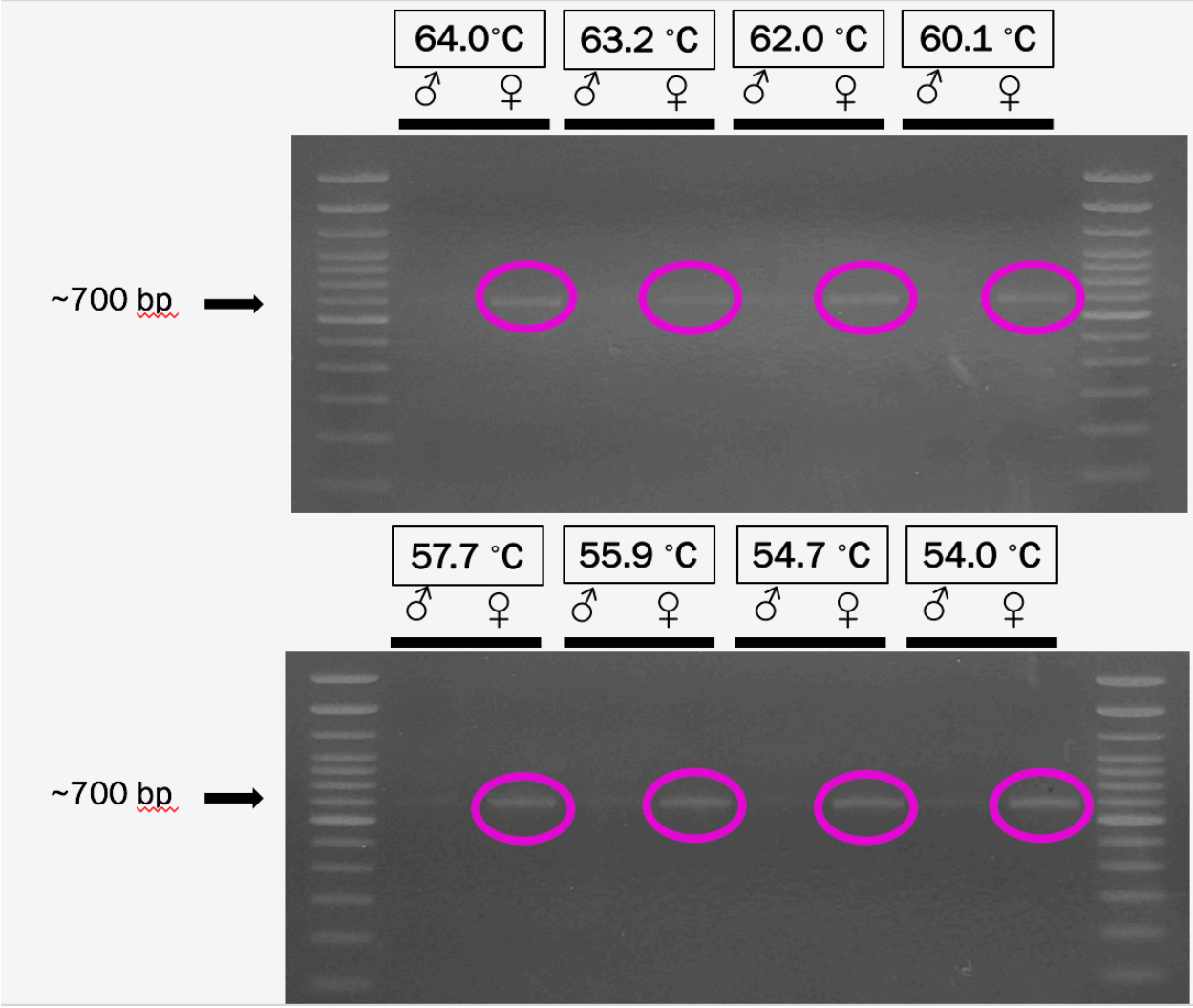

Figure 5.1. Agarose gel visualization of the gel product obtained from the first gradient PCR of species *C. rufifacies*. Female bands can be seen at 64.0 ºC, 63.2 ºC, 62.0 ºC, 60.1 ºC, 57.7 ºC, 55.9 ºC, 54.7 ºC and 54.0 ºC at approximately 700 base pairs and no distinct male bands are present at any temperature.

qPCR

All cDNA samples were analyzed on qPCR for statistical analysis and to complete an RT check to ensure no presence of genomic DNA. The housekeeper gene *rp49* was used to perform this check which has a known melt temperature of approximately 78.5 ºC (Faris 2017). All RT-

samples were negative for the presence of genomic DNA and did not have the presence of a melt temperature while all RT+ samples had a melt temperature at approximately 78.5 ºC. The results for this can be seen in Tables 5.2, 5.3 and 5.4, and the cycle fluorescence can be seen in Tables 5.5, 5.6 and 5.7. Tables 5.2 and 5.5 show the first cDNA conversion for the first replicate of these samples. A second replicate of cDNA for these samples was needed after consumption of the first replicate and these cDNA values can be seen in Tables 5.3 and 5.6. Lastly, A third replicate of cDNA for these samples was needed after consumption of the second replicate and these cDNA values can be seen in Tables 5.4 and 5.7.

Table 5.2. qPCR results for melt temperatures for the RT+ and RT- samples for replicate 1 *C. rufifacies* samples.

Sample	**Replicate**	**RT+ Melt Temperature (ºC)**	**RT- Melt Temperature (ºC)**
R1	1	79.5	None
R2	1	79.5	None
R3	1	79.5	None
R4	1	79.5	None
R5	1	79.5	None
R6	1	79.5	None
R7	1	79.5	None
R8	1	79.5	None
R9	1	79.5	None
R10	1	79.5	None

Table 5.3. qPCR results for melt temperatures for the RT+ and RT- samples for replicate 2 *C. rufifacies* samples.

Sample	Replicate	RT+ Melt Temperature (ºC)	RT- Melt Temperature (ºC)
R1	2	79.5	None
R2	2	80.0	None
R3	2	80.0	None
R4	2	80.0	None
R5	2	80.0	None
R6	2	80.0	None
R7	2	80.0	None
R8	2	80.0	None
R9	2	80.0	None
R10	2	80.0	None

Table 5.4. qPCR results for melt temperatures for the RT+ and RT- samples for replicate 3 *C. rufifacies* samples.

Sample	Replicate	RT+ Melt Temperature (ºC)	RT- Melt Temperature (ºC)
R1	3	80.0	None
R2	3	80.0	None
R3	3	80.0	None
R4	3	80.0	None
R5	3	80.0	None
R6	3	80.0	None
R7	3	80.0	None
R8	3	80.0	None
R9	3	80.0	None
R10	3	79.5	None

Table 5.5. qPCR results for cycle fluorescence for replicate 1 *C. rufifacies* samples.

Sample	Replicate	Cq Replicate 1	Cq Replicate 2	Cq Mean Replicate 1	Cq Mean Replicate 2	Cq Standard Deviation Replicate 1	Cq Standard Deviation Replicate 2
R1	1	20.69	20.66	20.67	20.67	0.021	0.021
R2	1	22.32	22.04	22.18	22.18	0.195	0.195
R3	1	21.97	-	21.97	-	0	-
R4	1	21.51	21.69	21.60	21.60	0.124	0.124
R5	1	21.84	21.64	21.74	21.74	0.138	0.138
R6	1	20.95	20.50	20.73	20.73	0.319	0.319
R7	1	20.81	20.76	20.78	20.78	0.037	0.037
R8	1	19.33	19.72	19.53	19.53	0.276	0.276
R9	1	20.39	20.15	20.27	20.27	0.166	0.166
R10	1	20.42	20.54	20.48	20.48	0.084	0.084

Table 5.6. qPCR results for cycle fluorescence for replicate 2 *C. rufifacies* samples.

Sample	Replicate	Cq Replicate 1	Cq Replicate 2	Cq Mean Replicate 1	Cq Mean Replicate 2	Cq Standard Deviation Replicate 1	Cq Standard Deviation Replicate 2
R1	2	22.51	22.39	22.45	22.45	0.081	0.081
R2	2	23.74	23.58	23.66	23.66	0.114	0.114
R3	2	23.58	23.40	23.49	23.49	0.126	0.126
R4	2	23.07	23.14	23.11	23.11	0.052	0.052
R5	2	23.27	23.08	23.17	23.17	0.135	0.135
R6	2	22.24	22.88	22.56	22.56	0.453	0.453
R7	2	21.72	21.45	21.58	21.58	0.1888	0.1888
R8	2	21.76	21.86	21.81	21.81	0.072	0.072
R9	2	22.16	22.40	22.28	22.28	0.173	0.173
R10	2	22.25	21.07	21.66	21.66	0.845	0.845

Table 5.7. qPCR results for cycle fluorescence for replicate 3 *C. rufifacies* samples.

Sample	Replicate	Cq Replicate 1	Cq Replicate 2	Cq Mean Replicate 1	Cq Mean Replicate 2	Cq Standard Deviation Replicate 1	Cq Standard Deviation Replicate 2
R1	3	22.63	22.79	22.71	22.71	0.112	0.112
R2	3	23.08	23.25	23.16	23.16	0.116	0.116
R3	3	22.91	22.75	22.83	22.83	0.113	0.113
R4	3	22.51	22.72	22.61	22.61	0.152	0.152
R5	3	22.96	23.04	23.00	23.00	0.054	0.054
R6	3	22.29	22.24	22.27	22.27	0.037	0.037
R7	3	21.43	21.84	21.63	21.63	0.286	0.286
R8	3	20.61	20.57	20.59	20.59	0.029	0.029
R9	3	21.35	21.10	21.23	21.23	0.175	0.175
R10	3	21.92	21.92	21.92	21.92	0.002	0.002

CHAPTER VI

ERROR RATES

Standard PCR

Lucilia sericata

For the first standard PCR, the annealing temperature 48.9 ºC was used. Twenty samples and a negative control were tested to determine the accuracy of the sex determination assay for *L. sericata*. As seen in Figure 6.1 and Table 6.1, the sex was determined for the samples. In lanes 7, 8 and 9 on the top and lanes 4, 8, 9 and 11 on the bottom, products of approximately 200 base pairs were visualized indicating that they are female samples. In lane 3 on top and lanes 2 and 7 on the bottom, products of approximately 500 base pairs were visualized indicating they are male samples. In lanes 2, 4, 5, 6, 10 and 11 on top and lanes 3, 5, 6 and 10 on the bottom, there is no product present. This indicates that there is likely an error that occurred somewhere in the methodology to where PCR product was not obtained. The negative control ran with PCR was negative. Of the 10 of 20 PCRs that yielded a result, all 10 were assigned to the appropriate sex.

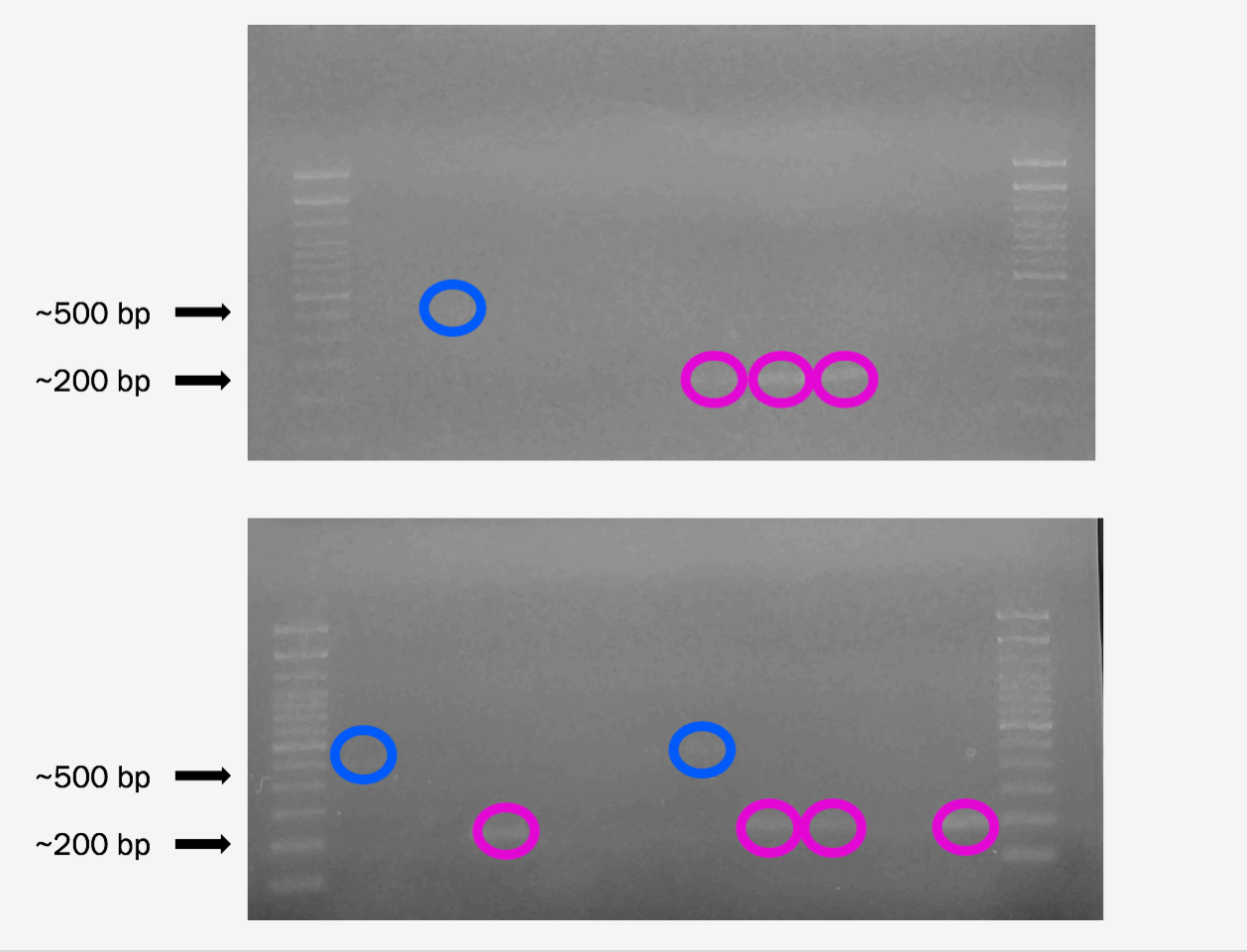

Figure 6.1. Agarose gel visualization of the gel product obtained from the first standard PCR of species *L. sericata*. The determination of males and females present is indicated by the presence of blue or pink circles respectively with males containing a 500 base pair product and females containing a 200 base pair product. A chart indicating the sex is below in Table 6.1.

Table 6.1. A summary table indicating the determination of sex from the *L. sericata* assay in comparison with the actual known sex of the sample. The accuracy of the assay is determined.

Sample ID	Sex Determined	Actual Sex	Correct
EL1	No bands present	F	Excluded
EL2	M	M	Yes
EL3	No bands present	F	Excluded
EL4	No bands present	M	Excluded
EL5	No bands present	F	Excluded
EL6	F	F	Yes
EL7	F	F	Yes
EL8	F	F	Yes
EL9	No bands present	F	Excluded
EL10	No bands present	M	Excluded
EL11	M	M	Yes
EL12	No bands present	F	Excluded
EL13	F	F	Yes
EL14	No bands present	M	Excluded
EL15	No bands present	F	Excluded
EL16	M	M	Yes
EL17	F	F	Yes
EL18	F	F	Yes
EL19	No bands present	F	Excluded
EL20	F	F	Yes

Cochliomyia macellaria

For the second standard PCR, the annealing temperature 57.7 ºC was used. Twenty samples and a negative control were tested to determine the accuracy of the sex determination assay for *C. macellaria*. As seen in Figure 6.2 and Table 6.2, the sex was determined for the samples. In lanes 2, 5, 7, 8 and 11 on the top and lanes 3, 5, 7, 10 and 11 on the bottom, products of approximately 200 base pairs were visualized indicating that they are female samples. In lanes 3, 4, 6, 9 and 10 on the top and lanes 2, 4, 6 and 8 on the bottom, products of approximately 350 and 500 base pairs were visualized indicating they are male samples. The sample in lane 9 on the bottom there is no product present. This indicates that there is likely an error that occurred somewhere in the methodology to where PCR product was not obtained. The negative control ran with PCR was negative. Of the 19 of 20 PCRs that yielded a result, all 19 were assigned to the appropriate sex.

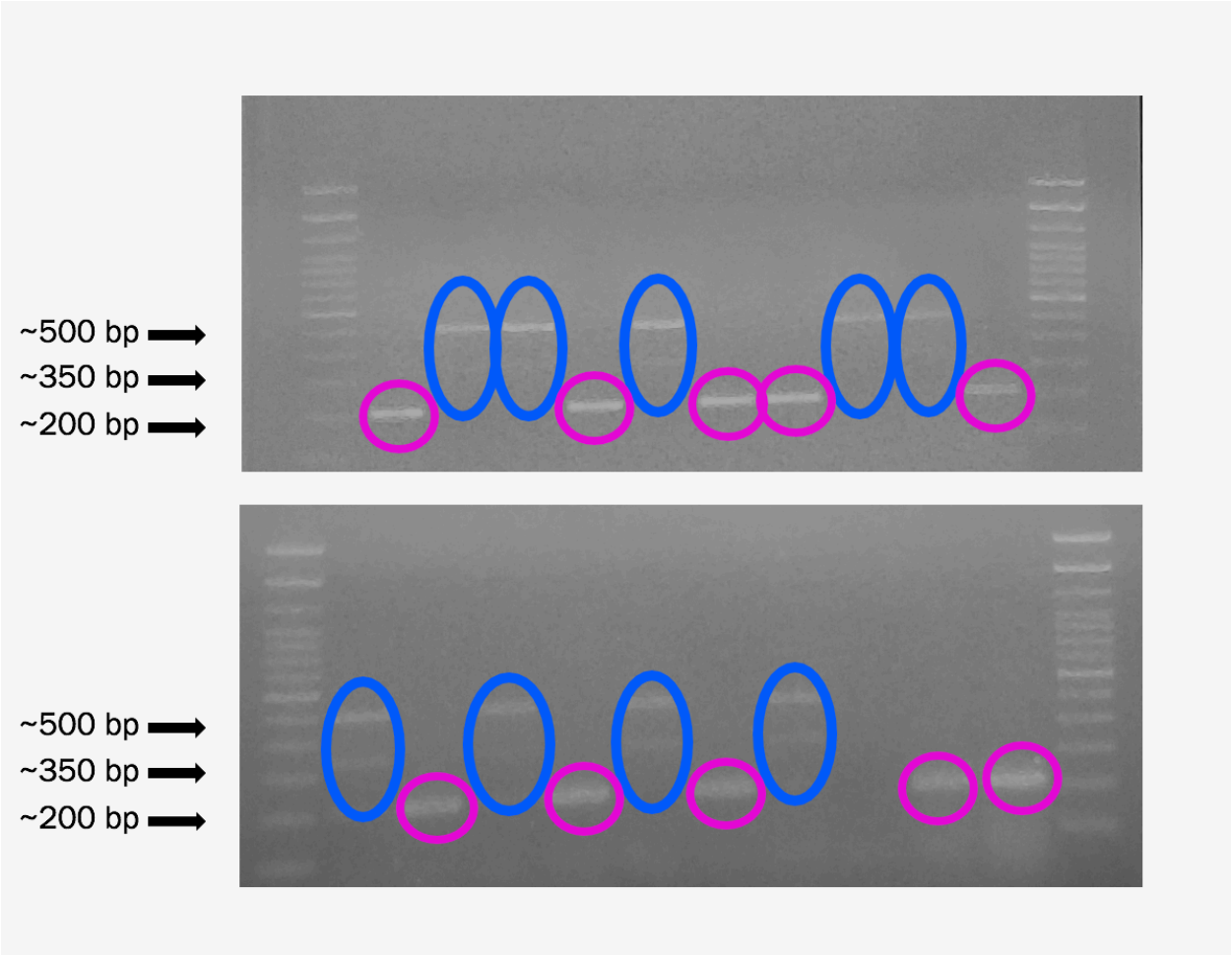

Figure 6.2. Agarose gel visualization of the gel product obtained from the second standard PCR of species *C. macellaria*. The determination of males and females present is indicated by the presence of blue or pink circles respectively with males containing 500 and 350 base pairs products and females containing a 200 base pair product. A chart indicating the sex is below in Table 6.2.

Table 6.2. A summary table indicating the determination of sex from the *C. macellaria* assay in comparison with the actual known sex of the sample. The accuracy of the assay is determined.

Sample ID	Sex Determined	Actual Sex	Correct
EM1	Female	Female	Yes
EM2	Male	Male	Yes
EM3	Male	Male	Yes
EM4	Female	Female	Yes
EM5	Male	Male	Yes
EM6	Female	Female	Yes
EM7	Female	Female	Yes
EM8	Male	Male	Yes
EM9	Male	Male	Yes
EM10	Female	Female	Yes
EM11	Male	Male	Yes
EM12	Female	Female	Yes
EM13	Male	Male	Yes
EM14	Female	Female	Yes
EM15	Male	Male	Yes
EM16	Female	Female	Yes
EM17	Male	Male	Yes
EM18	No bands present	Male	Excluded
EM19	Female	Female	Yes
EM20	Female	Female	Yes

Chrysomya rufifacies

For the third standard PCR, the annealing temperature 54.0 ºC was used. Twenty samples and a negative control were tested to determine the accuracy of the sex determination assay for *C. rufifacies*. As seen in Figure 6.3 and Table 6.3, the sex was determined for the samples. In lanes 2, 3, 7, 8 and 10 on the top and lanes 7, 8 and 9 on the bottom, products of approximately 700 base pairs were visualized indicating they are female samples. In lanes 4, 5, 6, 9 and 11 on the top and lanes 2, 3, 4, 5, 6, 10 and 11 on the bottom, no products were visualized. Due to the nature of the *dsx* gene and the different male and female exons, no bands present in the presence of a female primer is an indication of male samples. The negative control ran with PCR was negative. Of the 20 PCRs that yielded a result, 19 were assigned to the appropriate sex.

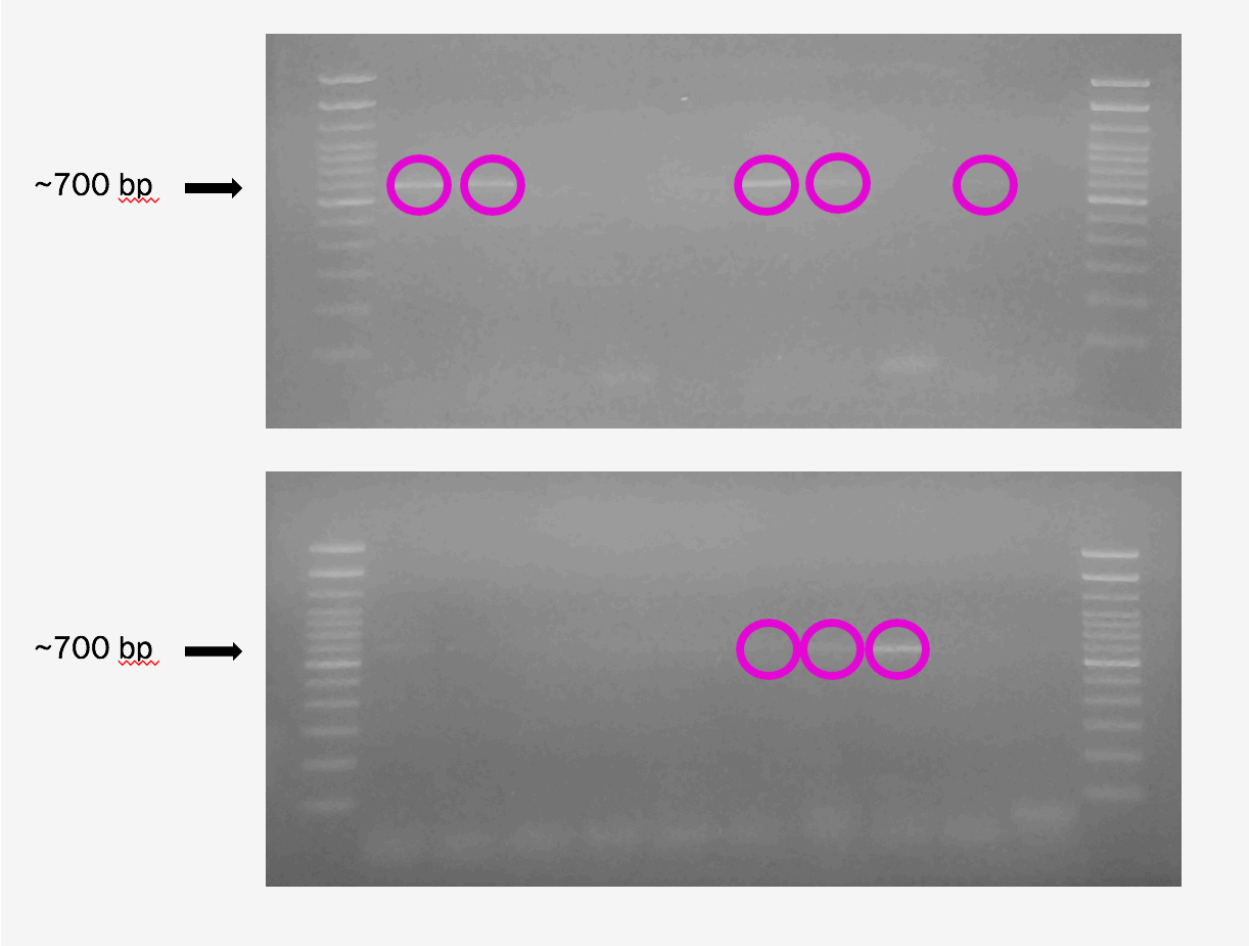

Figure 6.3. Agarose gel visualization of the gel product obtained from the third standard PCR of species *C. rufifacies*. The determination of females present is indicated by the presence of pink circles with females containing a 700 base pair product. The determination of males is indicated by the lack of bands present upon gel visualization. A chart indicating the sex is below in Table 6.3.

Table 6.3. A summary table indicating the determination of sex from the *C. ruifacies* assay in comparison with the actual known sex of the sample. The accuracy of the assay is determined.

Sample ID	Sex Determined	Actual Sex	Correct
ER1	F	F	Yes
ER2	F	F	Yes
ER3	M	M	Yes
ER4	M	M	Yes
ER5	M	M	Yes
ER6	F	F	Yes
ER7	F	F	Yes
ER8	M	M	Yes
ER9	F	F	Yes
ER10	M	M	Yes
ER11	M	M	Yes
ER12	M	M	Yes
ER13	M	M	Yes
ER14	M	M	Yes
ER15	M	F	No
ER16	F	F	Yes
ER17	F	F	Yes
ER18	F	F	Yes
ER19	M	M	Yes
ER20	M	M	Yes

qPCR

All cDNA samples were analyzed on qPCR for statistical analysis and to complete an RT check to ensure no presence of genomic DNA. The housekeeper gene *rp49* was used to perform this check which has a known melt temperature of approximately 78.5 ºC (Faris 2017). All RT- samples were negative for the presence of genomic DNA and did not have the presence of a melt temperature while all RT+ samples had a melt temperature at approximately 78.5 ºC. The results for melt curve and fluorescence can be seen in Tables 6.4 through 6.9 below. *L. sericata* melt curve data is in Table 6.4 and fluorescence data is in Table 6.5. *C. macellaria* melt curve data is in Table 6.6 and fluorescence data is in Table 6.7. *C. rufifacies* melt curve data is in Table 6.8 and fluorescence data is in Table 6.9.

Table 6.4. qPCR results for melt temperatures for the RT+ and RT- samples for *L.sericata* sex determination samples.

Sample	**Replicate cDNA sample**	**RT+ Melt Temperature (ºC)**	**RT- Melt Temperature (ºC)**
EL1	1	79.5	None
EL2	1	79.5	None
EL3	1	79.5	None
EL4	1	79.5	None
EL5	1	79.5	None
EL6	1	79.5	None
EL7	1	79.5	None
EL8	1	79.	None
EL9	1	79.5	None
EL10	1	79.5	None
EL11	1	79.5	None
EL12	1	79.5	None
EL13	1	79.5	None
EL14	1	79.5	None
EL15	1	79.5	None
EL16	1	79.5	None
EL17	1	79.5	None
EL18	1	79.	None
EL19	1	79.5	None
EL20	1	79.5	None

Table 6.5. qPCR results for cycle fluorescence for *L.sericata* sex determination samples.

Sample	Replicate cDNA sample	Cq Replicate 1	Cq Replicate 2	Cq Mean Replicate 1	Cq Mean Replicate 2	Cq Standard Deviation Replicate 1	Cq Standard Deviation Replicate 2
EL1	1	20.85	20.67	20.76	20.76	0.123	0.123
EL2	1	20.25	20.29	20.27	20.27	0.029	0.029
EL3	1	22.31	22.47	22.39	22.39	0.110	0.110
EL4	1	20.25	20.29	20.27	20.27	0.029	0.029
EL5	1	21.24	21.22	21.23	21.23	0.019	0.019
EL6	1	18.60	18.58	18.59	18.59	0.017	0.017
EL7	1	20.21	19.55	19.88	19.88	0.462	0.462
EL8	1	19.13	19.19	19.16	19.16	0.038	0.038
EL9	1	21.43	21.38	21.41	21.41	0.033	0.033
EL10	1	19.88	22.36	21.12	21.12	1.758	1.758
EL11	1	19.69	19.54	19.62	19.62	0.109	0.109
EL12	1	20.28	20.39	20.34	20.34	0.074	0.074
EL13	1	18.72	18.57	18.65	18.65	0.107	0.107
EL14	1	19.69	19.54	19.62	19.62	0.109	0.109
EL15	1	20.79	20.67	20.73	20.73	0.085	0.085
EL16	1	20.05	20.21	20.13	20.13	0.113	0.113
EL17	1	18.72	18.57	18.65	18.65	0.107	0.107
EL18	1	19.93	19.68	19.81	19.81	0.177	0.177
EL19	1	21.06	20.99	21.02	21.02	0.046	0.046
EL20	1	19.14	19.10	19.12	19.12	0.038	0.038

Table 6.6. qPCR results for melt temperatures for the RT+ and RT- samples for *C. macellaria* sex determination samples.

Sample	Replicate cDNA sample	RT+ Melt Temperature (ºC)	RT- Melt Temperature (ºC)
EM1	1	79.5	None
EM2	1	79.5	None
EM3	1	79.5	None
EM4	1	79.5	None
EM5	1	79.5	None
EM6	1	79.5	None
EM7	1	79.5	None
EM8	1	79.5	None
EM9	1	79.5	None
EM10	1	79.0	None
EM11	1	79.0	None
EM12	1	79.0	None
EM13	1	79.0	None
EM14	1	79.0	None
EM15	1	79.0	None
EM16	1	79.5	None
EM17	1	79.5	None
EM18	1	79.5	None
EM19	1	79.0	None
EM20	1	79.0	None

Table 6.7. qPCR results for cycle fluorescence for *C. macellaria* sex determination samples.

Sample	Replicate cDNA sample	Cq Replicate 1	Cq Replicate 2	Cq Mean Replicate 1	Cq Mean Replicate 2	Cq Standard Deviation Replicate 1	Cq Standard Deviation Replicate 2
EM1	1	22.77	22.23	22.50	22.50	0.385	0.385
EM2	1	22.98	22.91	22.94	22.94	0.043	0.043
EM3	1	22.32	22.23	22.28	22.28	0.069	0.069
EM4	1	22.78	22.66	22.72	22.72	0.086	0.086
EM5	1	22.08	21.97	22.03	22.03	0.078	0.078
EM6	1	22.65	22.66	22.65	22.65	0.010	0.010
EM7	1	23.38	23.37	23.37	23.37	0.001	0.001
EM8	1	23.37	23.51	23.44	23.44	0.097	0.097
EM9	1	25.54	25.48	25.50	25.50	0.058	0.058
EM10	1	23.11	23.30	23.21	23.21	0.134	0.134
EM11	1	23.38	23.50	23.44	23.44	0.080	0.080
EM12	1	20.77	20.84	20.81	20.81	0.045	0.045
EM13	1	22.79	22.91	22.85	22.85	0.089	0.089
EM14	1	23.62	23.02	23.32	23.32	0.424	0.424
EM15	1	22.13	22.05	22.09	22.09	0.056	0.056
EM16	1	21.60	20.76	21.18	21.18	0.596	0.596
EM17	1	21.27	21.17	21.22	21.22	0.074	0.074
EM18	1	24.24	22.05	23.15	23.15	1.548	1.548
EM19	1	25.67	25.14	25.41	25.41	0.369	0.369
EM20	1	N/A	N/A	N/A	N/A	N/A	N/A

Table 6.8. qPCR results for melt temperatures for the RT+ and RT- samples for *C. rufifacies* sex determination samples.

Sample	Replicate cDNA sample	RT+ Melt Temperature (ºC)	RT- Melt Temperature (ºC)
ER1	1	79.5	None
ER2	1	79.5	None
ER3	1	79.5	None
ER4	1	79.5	None
ER5	1	79.5	None
ER6	1	79.0	None
ER7	1	79.5	None
ER8	1	79.5	None
ER9	1	79.5	None
ER10	1	79.0	None
ER11	1	79.0	None
ER12	1	79.5	None
ER13	1	79.5	None
ER14	1	79.5	None
ER15	1	79.5	None
ER16	1	79.5	None
ER17	1	79.5	None
ER18	1	79.5	None
ER19	1	79.5	None
ER20	1	79.5	None

Table 6.9. qPCR results for cycle fluorescence for *C. rufifacies* sex determination samples.

Sample	Replicate cDNA sample	Cq Replicate 1	Cq Replicate 2	Cq Mean Replicate 1	Cq Mean Replicate 2	Cq Standard Deviation Replicate 1	Cq Standard Deviation Replicate 2
ER1	1	22.05	21.86	21.95	21.95	0.134	0.134
ER2	1	21.36	21.24	21.30	21.30	0.083	0.083
ER3	1	22.79	22.73	22.76	22.76	0.037	0.037
ER4	1	23.30	23.14	23.22	23.22	0.115	0.115
ER5	1	23.30	23.46	23.38	23.38	0.113	0.113
ER6	1	22.29	21.64	21.97	21.97	0.460	0.460
ER7	1	22.74	22.69	22.71	22.71	0.035	0.035
ER8	1	22.41	22.61	22.51	22.51	0.138	0.138
ER9	1	23.32	23.25	23.28	23.28	0.049	0.049
ER10	1	22.79	22.73	22.76	22.76	0.037	0.037
ER11	1	23.81	23.66	23.74	23.74	0.105	0.105
ER12	1	23.35	23.38	22.36	22.36	0.021	0.021
ER13	1	23.39	23.20	23.30	23.30	0.129	0.129
ER14	1	22.68	23.04	22.86	22.86	0.253	0.253
ER15	1	23.76	23.65	23.70	23.70	0.078	0.078
ER16	1	23.07	23.06	23.06	23.06	0.011	0.011
ER17	1	22.05	21.86	21.95	21.95	0.134	0.134
ER18	1	21.36	21.24	21.30	21.30	0.083	0.083
ER19	1	22.41	22.61	22.51	22.51	0.138	0.138
ER20	1	22.29	21.64	21.97	21.97	0.460	0.460

CHAPTER VII

DISCUSSION

Implications in Forensic Science

It is known that insects, and more specifically blow flies, have differing development time between males and females (Honek 1997). When blow flies are found on remains, adult specimens can be sight identified for sex while immature forms have no standardized method for sex identification (Whitworth 2006). Knowing there is a differing development time for males and females, but not having a standardized method for sexing immature forms at a remains recovery site can lead to uncertainty in time of colonization (TOC) estimates as differences between sexes are not being accounted for (Smith and Wells 2016). For this reason, I posed the question; "Can I develop a method and optimize an assay for determining sex of immature blow flies for forensic applications?" The creation of this assay will provide a method for identifying immatures found on remains and therefore allow forensic entomologists to account for differences in development times between male and female blow flies. Applying this knowledge will reduce the uncertainty within TOC estimates and allow for more precise estimates. This assay will also aide in our understanding of gene expression and how males and females may give rise to differing levels of expression for genes.

Sex Determination *Lucilia sericata*

In *L. sericata*, the transformer gene plays a very important role in sex-specific phenotypes that may arise. This can be seen in the sex-determination pathway in Figure 7.1 below where the *transformer* gene (*tra*) is at the top of the hierarchy for sex-determination.

Therefore, when designing primers, the *tra* sequence was targeted for primers that would allow for determination of sex.

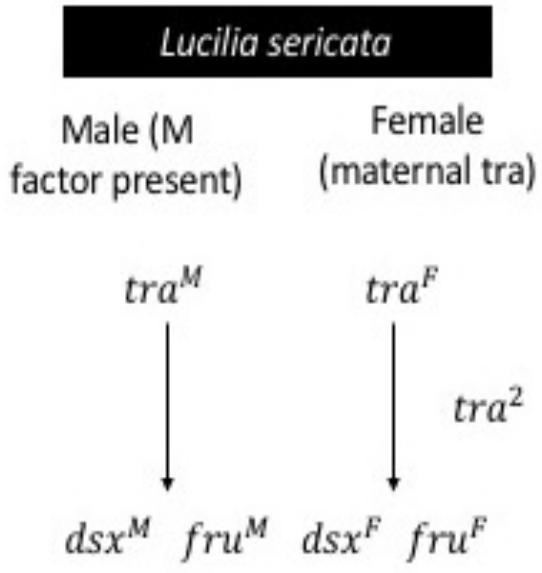

Figure 7.1. Sex determination mechanism of *L. sericata*.

Accession number JX315620, *L. sericata tra* gene, was used to find a forward and reverse primer target for the *tra* gene. As seen in Figure 7.2, forward primer 5'-ATT TAA AAT TCA ACA ATC CAT ACC C-3' is nucleotide 98-122 contained within the first exon of the *tra* gene sequence. The reverse primer 5'-TCT AAA TTA TTA GTA TCA CGA GCA T-3' is nucleotide 2659-2683 within the second exon of the *tra* gene sequence. From this depiction, we know that between the forward and reverse primer lies a portion of exon 1, a male exon, an intron and a portion of exon 2.

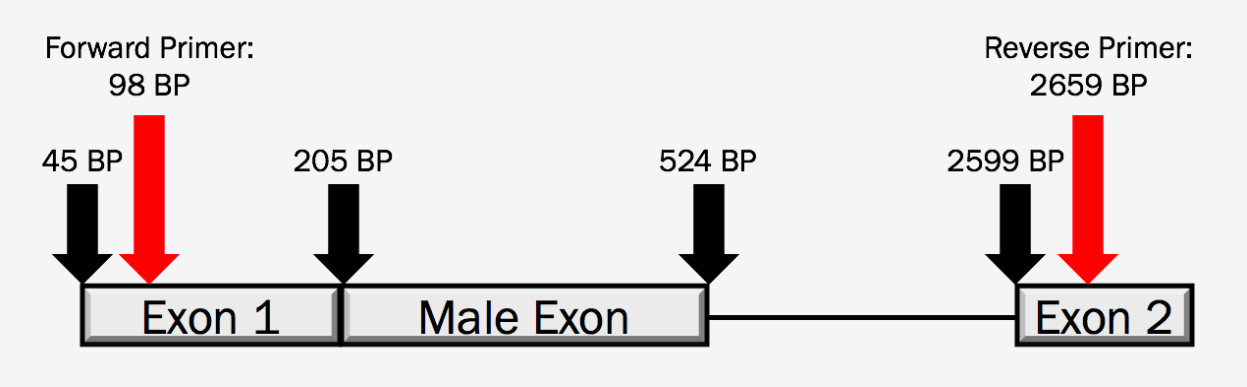

Figure 7.2. *tra* sequence for *L. sericata* as constructed from NCBI accession number JX315620.1. The forward and reverse primer selected are indicated within the sequence of *tra* by red arrows. The line in between the male exon and exon 2 is an intron.

In Figure 7.3, within the splicing product, females will contain the portion of exon 1 and the portion of exon 2, but not the male exon or the intron. Calculated out, there is an expected product of 193 base pairs for females, which confirms a correct product length from our assay where females produced ~200 base pair splicing product. For males, their splicing product will contain the portion of exon 1, the portion of exon 2 and some variant of the male exon, but not the intron. Calculated out, there is an expected product of 511 base pairs for males, which confirms a correct product length from our assay where males produced ~500 base pair splicing product. Of the 10 samples that yielded PCR product, all 10 were assigned to the appropriate sex, yielding great accuracy for this assay. However, some focus needs to be on yielding better bands for this species. From Li et al. 2013, the expected splicing product length for females was 799 base pairs and for males 1,118 base pairs. However, as seen from our assay, using the same primers we obtained a splicing product length of approximately 200 base pairs for females and approximately 500 base pairs for males. Our splicing product lengths are further strengthened by the calculation based on the published transcriptome from accession number JX315620 (Sze

2012). It is believed that the difference in splicing product length may arise from the confusion in the primer pair being used for gel visualization in the Li et al. 2013 paper. As multiple primer pairs are used within the paper, it appears the same primer pair was used for gel visualization, however it is not explicitly clear.

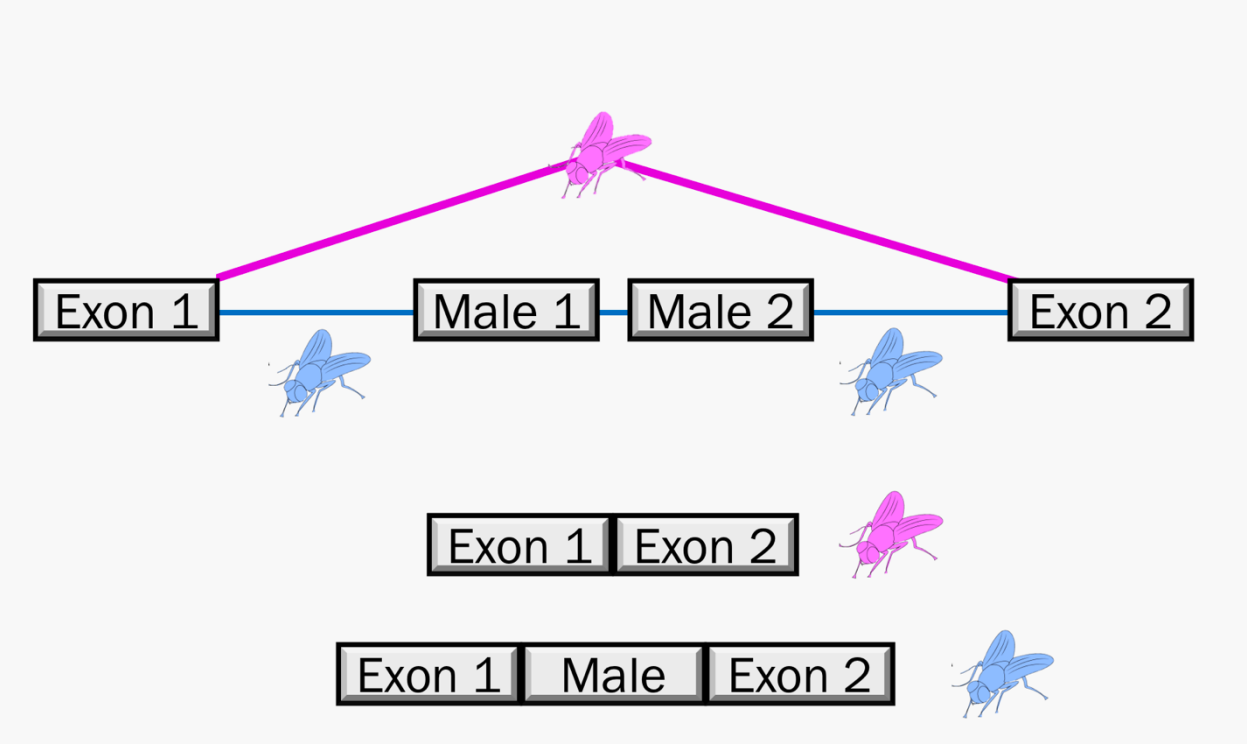

Figure 7.3. Differential splicing pathway for *L. sericata*, whereby male splicing products will be longer than female splicing products. Female splicing products will only contain exon 1 and exon 2, but neither the male exon nor the intron sequence. Male splicing products will contain exon 1, exon 2 and some variation of a male exon, but not the intron sequence. For this reason, males will have a longer splicing product than females.

Sex Determination *Cochliomyia macellaria*

In *C. macellaria*, *tra* plays a very important role in sex-specific phenotypes that may arise. This can be seen in the sex-determination pathway in Figure 7.4 below where *tra* is at the top of the hierarchy for sex-determination. Therefore, when designing primers, the *tra* sequence was targeted for primers that would allow for determination of sex.

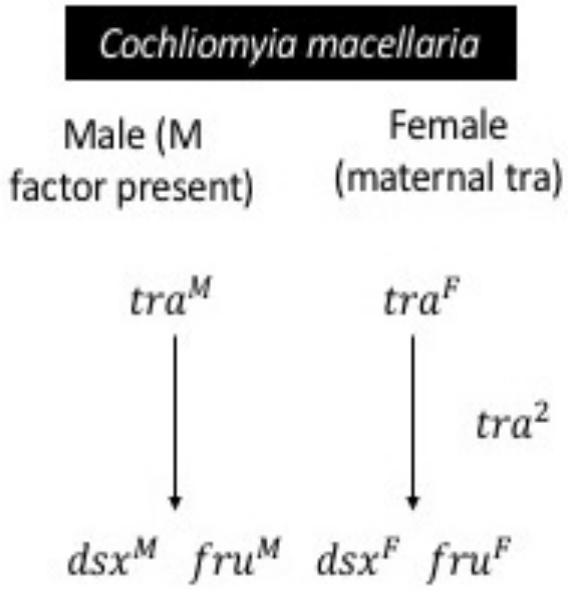

Figure 7.4. Sex determination mechanism of *C. macellaria*.

Accession number JX315619, *C. macellaria tra* gene, was used to find a forward and reverse primer target for the *tra* gene. The primer pair containing the forward primer 5'-CAT GCA ATT GTG CGT TCG GT-3' and reverse primer 5'-CGT CTT CTT CTT GGC GGA CT-3' were tested first but did not work. A different primer set contained within the gene was tried and did yield results. As seen in Figure 7.5, forward primer 5'-ATA CCA AGT GGT TCG GTG AAA

AGA GGT C -3’ is nucleotide 73-100 contained within the first exon of the *tra* gene sequence. The reverse primer 5’-TCT AAA TTA TTA GTA TCA CGA GCA T-3’ is nucleotide 4618-4647 within the second exon of the *tra* gene sequence. From this depiction, we know that between the forward and reverse primer lies a portion of exon 1, a male exon, an intron and a portion of exon 2.

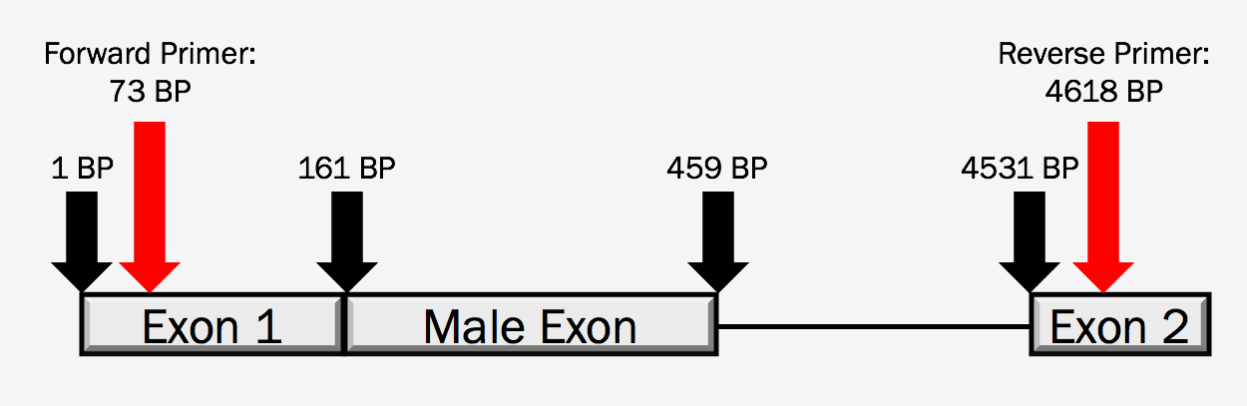

Figure 7.5. Transformer gene sequence for *C. macellaria* as constructed from NCBI accession number JX315619.1. The forward and reverse primer selected are indicated within the sequence of the transformer gene by red arrows. The line in between the male exon and exon 2 is an intron.

In Figure 7.6, within the splicing product, females will contain the portion of exon 1 and the portion of exon 2, but not the male exon or the intron. Calculated out, there is an expected product of 204 base pairs for females, which confirms a correct product length from our assay where females produced ~200 base pair splicing product. For males, their splicing product will contain the portion of exon 1, the portion of exon 2 and some variant of the male exon, but not the intron. Calculated out, there is an expected product of 502 base pairs for males, which

confirms a correct product length from our assay where males produced ~500 base pair splicing product. The males for *C. macellaria* also produced another splicing product band at ~350 base pairs which is likely due to a variation within the length of male exon contained by the male (Smith and Wells 2016). Of the 19 samples that yielded a result, all 19 were assigned to the appropriate sex, yielding great accuracy for this assay. From Li et al. 2013, the expected splicing product length for females was 779 base pairs and for males 1,077 base pairs. However, as seen from our assay, using the same primers we obtained a splicing product length of approximately 200 base pairs for females and approximately 500 base pairs for males. Our splicing product lengths are further strengthened by the calculation based on the in-house transcriptome from accession number JX315619. It is believed that the difference in splicing product length may arise from the confusion in the primer pair being used for gel visualization in the Li et al. 2013 paper. As multiple primer pairs are used within the paper, it appears the same primer pair was used for gel visualization, however it is not explicitly clear.

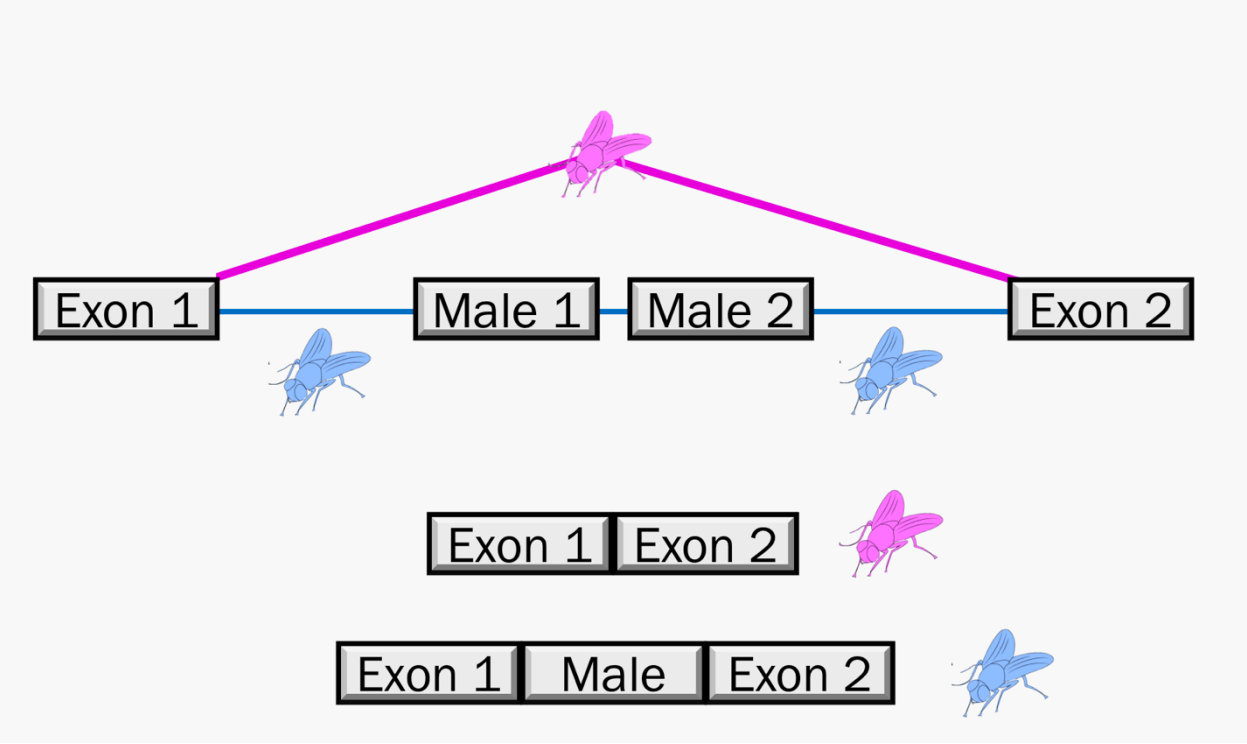

Figure 7.6. Differential splicing pathway for *C. macellaria*, whereby male splicing products will be longer than female splicing products. Female splicing products will only contain exon 1 and exon 2, but neither the male exon nor the intron sequence. Male splicing products will contain exon 1, exon 2 and some variation of a male exon, but not the intron sequence. For this reason, males will have a longer splicing product than females.

Sex Determination *Chrysomya rufifacies*

In *C. rufifacies*, the *doublesex* (*dsx*) gene rather than the *tra* gene plays a very important role in sex-specific phenotypes that may arise. In Figure 7.7 below, we can see that the gene involved in the sex determination for this species is not known however, *dsx* is believed to be conserved (Pimsler 2015). Therefore, when designing primers, the *dsx* gene sequence was targeted for primers that would allow for determination of sex.

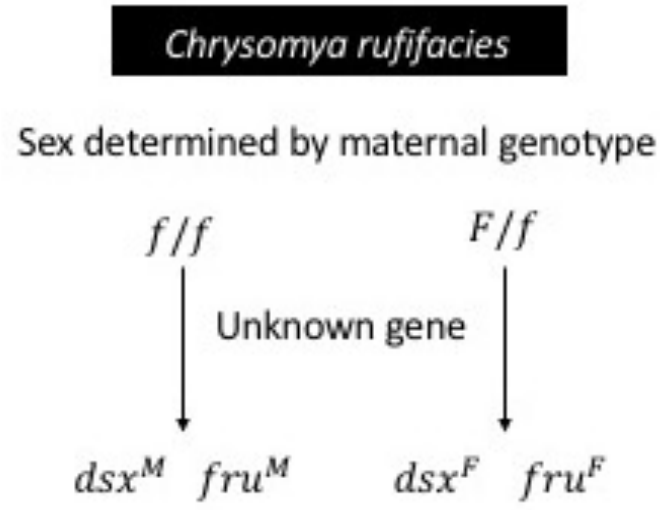

Figure 7.7. Sex determination mechanism of *C. rufifacies*.

In *C. rufifacies* the sex mechanism differs from *L. sericata* and *C. macellaria*, as this species has monogenic sex determination (Ullerich 1983). This means that only all male (arrhenogenic) or all female (thelygenic) offspring will laid based on the phenotype of the mother (Ullerich and Schottke 2006) (Figure 7.7). For this reason, two different sequences were targeted to determine males and females within this species. A splicing product for *C. rufifacies* will contain a common exon that both males and females will share and then males will contain a male exon, while females will contain a female exon (Sze et al. 2017) (Figure 7.8).

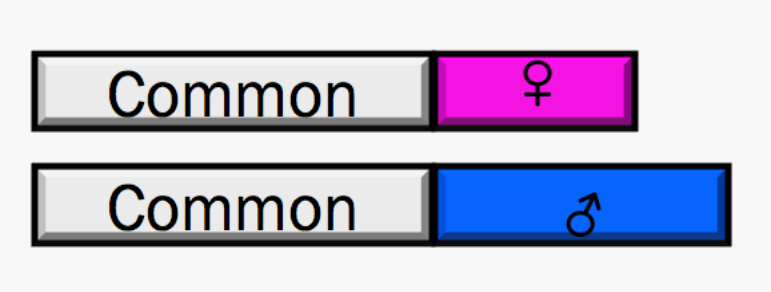

Figure 7.8. Differential splicing pathway for *C. rufifacies*, whereby male splicing products will be different than female splicing products. Female splicing products will contain the common exon and the female exon while male splicing products will contain the common exon and the male exon. For this reason, separate reverse primers are used for female and male samples.

Previously published transcriptome construction by Sze et al. 2017, identified a common exon, a male exon and a female exon for *C. rufifacies* (Figure 7.8). The common exon shared among *C. rufifacies* is 1573 base pairs long, the female exon is 117 base pairs long and the male exon is 876 base pairs long (Sze et al. 2017) (Figure 7.9). The common exon was targeted for a forward primer, sequence 5'- GCC ATG TTC CTG CTG CTC TA-3'. This primer sequence starts at nucleotide 975 within the common exon. For the reverse primer, a separate primer for males and females was targeted. A female reverse primer with sequence 5'-CAC ATT GTC GGG TGG CAC AA-3' is nucleotide 66- 86 within the female exon. Therefore, a splicing product of 684 base pairs is expected. This corresponds to the results from the assay presented as the splice product obtained was ~700 base pairs. A male reverse primer was not able to be optimized for the present assay. Though no male reverse primer could be optimized, there is still usefulness in this assay as absence of a band is indication of a male sample. The accuracy of the assay can be seen in 19 of 20 samples being correctly assigned to the appropriate sex.

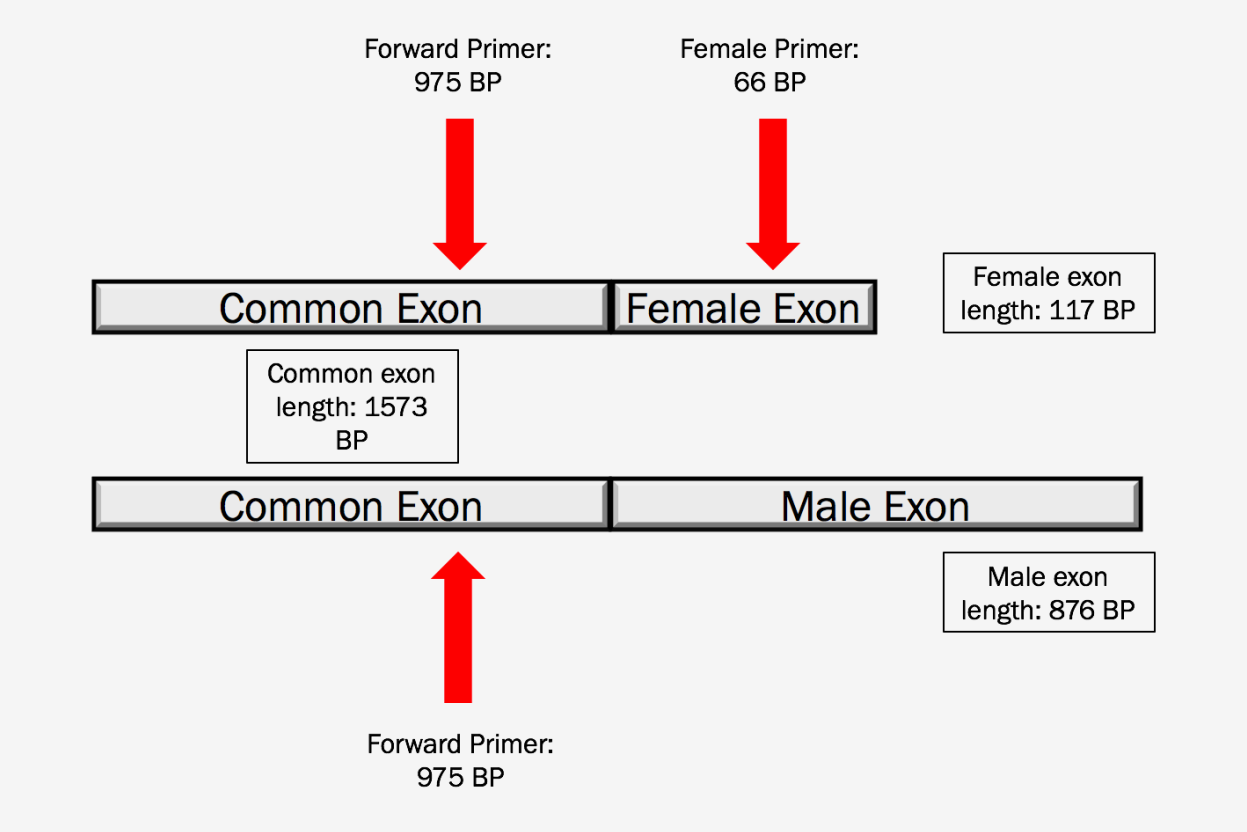

Figure 7.9. *dsx* gene sequence for *C. rufifacies* as constructed from node sequences noted in Appendix Figure 1.1, Appendix Figure 1.2 and Appendix Figure 1.3. The forward primer selected in the common exon and the female reverse primer selected in the female exon are indicated within the sequence of the *dsx* gene by red arrows.

Implications in Other Applications

In this study, the family Calliphoridae was of primary focus. Calliphoridae is a large and diverse family of insects that encompasses the three species *L. sericata*, *C. macellaria* and *C. rufifacies* (Sabrosky et al. 1999, Rognes and Pape 2007, 1998, Kurahshi 2007). All three of these species are located within the United States and more specifically the state of Texas (Rueda et al. 2010, Owings et al. 2014, Flores et al. 2014, Faris 2017). These three species also have varying importance within different facets of entomology. Some have medical importance in entomology

for wound debridement therapy in the regeneration of skin (Sherman 2009, Kerridge et al. 2005). Some have veterinary importance in entomology as they are known agents of myiasis in sheep better known as sheep strike (Wall 1995, Erzincliogl u 1987). Lastly, all three species have incredible importance in forensic entomology where they are used to calculate time of colonization estimates for forensic investigations (Greenberg 1991, Liu and Greenbe 1989). These three species were chosen for this study for their importance in forensic investigations. Though all three species have this implication in forensic investigations and this was the focus of this study, they also all have implications in other varying fields of entomology. In similarity, this assay has applications within these species and others for varying questions that wished to be asked throughout differing fields or applications of entomology.

Veterinary Importance

One such application is in veterinary entomology where species such as *C. homnivorax* and *L. cuprina* that cause myiasis in sheep known as sheep strike cause thousands of dollars in damage to livestock (Heath and Bishop 2006, Wardhaugh et al. 2007). To aide in ridding of these pest species, male sterilization has been a common solution to solving this problem. There are several ways that people have gone about this such as the sterile insect technique (SIT), creation of transgenic, male-only strains and more recently the use of sex-specific splicing to develop male-only strains. (Dyck et al. 2005, Li et al. 2014, Li et al. 2013) The technique of SIT, though incredibly successful, costs millions of dollars each year to control these pests (Vargas-Teran et al. 2005). A potential problem that occurs through SIT is that colonies reared for mass release may incur breakdown due to recombination events (Franz 2005). Other problems include sorting through large amounts of pests to determine whether they are male and female and the

time and money incurred through this, and the amount of radiation used in this technique sometimes kills the pest rather than allowing it to be sterile for release. More importantly, suitable mutations and rearrangement found in chromosomes for one species are not always transferable to another. Methods of engineered insect development allow for a differing approach for the genetic control of pests (O'Brochta and Handler 2008). These methods allow for the creation of male-only strains that give a different approach to ridding of these pests in a more cost-effective manner. With these male-only strains there can be release of these species into the area for eradication and mating with this male-only species will have the same effect as the SIT technique with some of the problems resolved. The creation of male-only strains requires no additional time or money spent sifting through pests to identify sex for radiation treatment, which eliminates the possibility of too much radiation exposure leading to death rather than sterilization. This method also allows for the creation of transgenic male-only strains in other similar species as it can also be readily transferred to related species (Scott et al. 2004). *tra* is a highly-conserved gene located within the sex determination pathway (Concha and Scott 2009). Utilizing this gene, male-only progeny can be generated and allow for the ability to mate by a molecular approach (Li et al. 2015). This was observed through the isolation of *tra* in Li et al. 2013 and then utilization of this target to create a transgenic sexing system in Li et al. 2014. This approach again is incredibly useful as the sex-specific splicing that occurs is highly conserved in at least three other species of calliphorids, including the commonly known pest *C. homnivorax* (Li et al. 2013). For this reason, this assay is useful in veterinary applications as it can target the *tra* sequence of pest species and then these targets can be used to create male-only strains useful for transgenic sexing system creation.

Ecological Applications

Sexual dimorphism is a very important source of phenotypic variation in an organism (Benitez 2013) and morphological characteristics arising from sexual differences are common across many taxa (Nunez-Rodriguez and Liria 2017). These differences in phenotypic variation occur in insects from body color in the ponenne ant and cuticular hydrocarbon expression in decorated crickets (Miyazaki 2014, Weddle 2012). Sex chromosomes are significant in their evolutionary importance and yield advantages and costs as well (Rice 1984, Feigel et al. 2009) One such characteristic, sexual dimorphism, played a very important role in this project as sex-specific differences will arise in blow fly species (Tabugo 2015, Hu et al. 2010).

Within the sex determination pathway, differential splicing plays an important role. This process occurs during gene expression where a gene will encode for several proteins. The male and female isoforms expressed from these proteins will give rise to sex-specific phenotypes. Examples of sex-specific phenotypes include things such as pigmentation in Drosophila (Gaunt and Paul 2012) and antennae in mosquitoes (Petrella 2014). The present assay is informative into the background of sexual dimorphism that is experienced within insects and will also lead to a better understanding of gene expression. With the help of this assay, it may be possible to identify genes that are more or less expressed in males or females, and lend information into why males and females will inherently gain a certain trait over another.

Future Work

Lucilia sericata

Future work to be done on this assay is to nest PCR primers to allow for better amplification of male bands for this primer set. Male bands upon gel visualization are faint and a

nested PCR reaction may allow for better visualization of splicing products. This may also yield in better results when trying to calculate error rates for the assay, as samples that may appear to have no bands may actually have bands present upon use of the nested primer set.

Other work is to apply this research to a known set of *L. sericata* samples where gene expression data based on age is currently known. With sex determination of these samples, it can be evaluated whether there is a difference in gene expression dependent on the sex of a blow fly at various ages. This will also be used in conjunction with a published transcriptome for this species providing an overall view for this species and the role sexual dimorphism plays in it.

Cochliomyia macellaria

For the species *C. macellaria*, the sex determination assay will be used to proactively restrict the testing of gene expression data to females or males only. This will allow for an overall view into the gene expression that this specific species will have and how sex may play a role within that. This species has a published transcriptome that can be used in totality with the other information to give an overall view into this species and gain a better understanding of sexual dimorphism.

Chrysomya rufifacies

Future work to be done on this assay, is to continue working toward a male reverse primer that can be used for testing the presence of male samples. If this occurs, it will then be possible to multiplex the common forward primer, the female reverse primer and the male reverse primer together so only a single PCR reaction will be needed when testing samples.

For the species *C. rufifacies*, the sex determination assay will be used to proactively restrict the testing of gene expression data to females or males only. This will allow for an overall view into the gene expression that this specific species will have and how sex may play a role within that. This species has a known transcriptome that can be used in totality with the other information to give an overall view into this species and gain a better understanding of sexual dimorphism.

CONCLUSION

We know there are differences between male and female insect development times, but there is currently no standardized method to identify sex of immature blow fly forms found at crime scenes (Honek 1997). Not accounting for this difference in development time causes time of colonization (TOC) estimates to have more uncertainty within them. For this reason, I posed the following question: can I develop a method and optimize an assay for determining sex of immature blow flies for forensic applications?

The present assay was found to be effective and reproducible in correctly identifying sex in three blow fly species of forensic importance-*Lucilia sericata, Cochliomyia macellaria and Chrysomya rufifacies*. Using known primer sets for *tra* and *dsx* genes, assays were created and optimized for use in forensic investigations. The assays proved to be reproducible with *L. sericata* yielding 10 of 10 appropriately assigned sexes, *C. macellaria* yielding 19 of 19 appropriately assigned sexes, and *C. rufifacies* yielding 19 of 20 appropriately assigned sexes..

The creation of this assay will provide a method for identifying immatures found on remains and therefore allow forensic entomologists to account for differences in development times between male and female blow flies. Applying this knowledge will reduce the uncertainty within TOC estimates and allow for more precise estimates. This assay will also aide in our understanding of gene expression and how males and females may give rise to differing levels of expression for genes.

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APPENDIX

Figure 1. *C. rufifacies* common exon sequence contained within the *doublesex* (*dsx*) gene that was used for primer creation. The sequence is approximately 1573 base pairs long.

5’AAAAAAAAAAGAAACAATGGATGATCGCAATTGTACATAAATGAAAAAATAAGA

AAAATCTGCTTAATAAATAAAAAAAGGAAAATAGCGTAAAGTAAATTTTTCAATCT

ATACAATGTAAAACACAAAAATTTACTGAAGTTTGAACAAGAAAATTCCAAAAAGA

GTAAAAACTTTWAAAACAACTTTTTAATATTTTTGAAGAGAGGCTGAAAAAAACTA

ACATTTTATTCATCTTATCTAGTCCATCTTATCGCACACCCGATAAAGAAAAAAACA

CATTAAACGTGTCAACTGGGTTTGAAATTTTTCATTTCGTTACTGAGTCGTTTGTTTT

TTTTTGTATACGCTTTGTTTATTCTTAAAGCGCTCAACAACACTGTTGGCTATTTTTCA

GCTGCAAAGAAAAAAATATATATAACATTTTTCGAATTTTCCAACACACCGTTGCCA

GTAACAGATTTTGAATGGTTTCTGAGGATACCAATTGGAATAGCAGTGACACAATGT

CCGACACGGACATGCACGATTCCAAAAATGATATTTGTGGTGGTGCATCCAGCTCTA

GTGGCAGCTCTGGCACCCCACGAACTAAACCGAATTGTGCACGTTGCCACAATCATG

GTTTTAAAATCAAATTAAAAGGCCACAAACGTTATTGTAAATTTCGCAATTGYAATT

GTGAGAAATGTCGCCTAACCGCTGATCGTCAGCGTGTCATGGCCTTACAGACGGCAC

TAAGACGTGCCCAGCAACAGGATGAACAACGTATATTACAGATGCATGAAGTRCCG

CCAGTTGTACATCCACCCACAGCCTTACTTAAGGCTCACTACCATCATCATCATCAG

TTACAACATCATATTTCCGAACAATTACATCACCATCATCATCCACATTTGGTGGAC

GCCGCTGCAGTTGTAGGTGCTGTACCTCCACATCATCCTCATCATCCACATCACCAT

CATCATGTGAGCCATGTTCCTGCTGCTCTAACAACAATACGTTCACCACCCCACAGT

GATCATAGTGCCAACGGTGGTTCAAGTACTGGTGGAAGTGGGGGTGGCGGTGGTGG

TGGTAGTGGTGCTTCTAATAGCGGTGGTGGTGGCGGTGGTATAGTTGGTAGTAGTGG

TAGTGTTATTGAACGAAATTCAGCTGCCGCTCTKAATGGTATGGCTAGTAGCAGCAG
TGTTGCTTCAAGTTCAACAGTTGGTCCACCACATCATTCGTCACCGGATCAAAATCA
ACATCATCATAATGCTCATCATCATCACTCACATTCACATTTATCAACAATGCCCTCA
ACTGCACAGTCTGTGGATTCATCTTGTGATTCGTCGTCACCATCACCCTCCTCAACAT
CGGGAGCTGTATCGTTGCCAGTTAATCGGAAACCTGTACCGGAGCATCATCAAAATG
GTGCTGATATGTCTATAGATCTAATATTAGATTATTGCCAAAAATTGATAGAAAAAT
TTGGTTATCCCTGGGAAATGATGCCTCTRATGTATGTGATACTAAAGGATGCTGGTG
TAGATATCGATGAGGCTTCAAAACGTATTGAAGAAGG3’

Figure 2. *C. rufifacies* female exon sequence contained within the *dsx* gene that was used for primer creation. The sequence is approximately 117 base pairs long.

5’ACAACATGTTGTTAATGAATATTCACGTCAACACAATTTAAATATTTATGATGGGT
GTGAATTACGTTGTGCCACCCGACAATGTGGTTGATAAATCTGAATATAAAAGATTT
ACAA3’

Figure 3. *C. rufifacies* male exon sequence contained within the *dsx* gene that was used for primer creation. The sequence is approximately 876 base pairs long.

5’CGTACAAGTCTTAAAACAATACTATACACTATTAAACATGTACGAGGGTAATGAA
TTGCGAAATAAATTTAAATTAAATCGGCGTCCAGATAATCCTGTTCCCGAAACAACC
AGAACAGAATGTGATGAAACAACCAAACGTATAAGACTAGAGGCTACTGAACAATT
AAATCAATTAACACAAACGTACTATAATTATCAACGTTATGCCACCCTACCGCCAGC
ATATTGGWCTTATCCATCAATACAATTTGGACGAGCTATATGGACGGAATTGCCAA
ATCCACATTTTGCTGCCGCAATAATACCACCACATCCAGCAACAACACCACCAGAGC

CAACAACACTTAGTCGACGTTCACCGAGTCCATCCAAAGTTAGTCAATCGGGTAGTA

GTATAGGTGGTGAATCTATAACAGCTAACACAACACCAACACCAACGATAAAAACA

TCAACTACAATACCTACTGCTGGCGTTATAGCCGCAGCAGCAGCAGCGGCAGCTGC

CGCCGCAGCAACGTAGCAACAATATCAAAATAAATTAAATGCAGCTGCTGTTACAA

ATGAAACTGAAACAGAAACAACGACGTCATCAGTTAATGCGGCGGCAGCAACTGTC

ATTTTACACGTAGATGATTAATTTCTGAAATTATTTACATTTGTAAGTAAATGTACGG

GTCTCTTAATAAATGAAAAATATTGAATAGCTGCTTATTCTAAAAAACCATGTACAA

CTAAAGAAAAATCTATAGCATAAGAACAATGTCAATACAGAATCTAACCTTAAGAT

GATCAAATTACAATTATTGATGAAAACTAAAAACTAACTTATGCTAACGTGATAAAA

ATCGTTATGGTTTTTGTATAGAATTTTCCC3’