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Testing Evolutionary Conservation of Sex Determination in Lepidoptera Using CRISPR/Cas9 Gene Editing

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by

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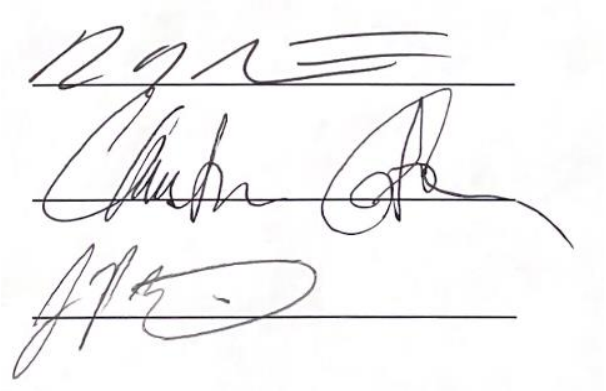

CONTENTS

ABSTRACT

The mechanism of sex determination in Lepidoptera is largely unexplored, and limited to knowledge of only a few genes. Adult Lepidoptera have obvious sexually dimorphic qualities, such as their genitalia. However, significant sex determination genes are not imperative for the adult developmental stage. Knockouts of some sex determination genes (*doublesex* isoforms) are shown to be lethal in the embryonic and larval stages of development in several animals. However, it is unclear whether this is due to their respective impact on sex determination and sexual dimorphism, or the existence of another function, such as pleiotropy, within a particular gene. Moreover, Little is known about the specific impacts of sex determination gene knockouts on Lepidopteran morphology and behavior. This honors thesis explores the effects of knockouts of key sex determination gene *masculinizer* (*masc*) in Lepidopteran species *Vanessa cardui*. *masc* is thought to be an important gene in some Lepidopteran species, specifically for the persistence of masculinization during embryonic development, and for dosage compensation of chromosomal activation on the Z chromosome. *masc* expression was knocked out in *Vanessa cardui* embryos using CRISPR/Cas9 gene editing which targeted the *masc* gene at two different positions within the *V. cardui* protein-coding sequence. Surviving adult butterflies were observed in order to determine phenotypic differences in sexually dimorphic anatomy and behavior. Specifically, vestigial forelegs were collected from each injected individual and analyzed for mosaic phenotypes. It was found through statistical and morphometric analysis that knockouts of the *masc* gene have the potential to decrease embryonic survival probability and adult fitness in *Vanessa cardui*, and produce mosaic phenotypes in surviving male animals. These findings support the hypothesis that *masc* is conserved across Lepidoptera, including *Vanessa cardui.*

CHAPTER ONE

INTRODUCTION

1.1: Sexual Dimorphism and Sex Determination

Sexual dimorphism is a common evolutionary process that occurs among earth's numerous animalia. The ultimate effect of sexual dimorphism is to distinguish males and females of a certain species. Sexually dimorphic features in animals may include anatomy of the reproductive organs, body size and weight, lifespan and mortality, and coloring(Encyclopedia Britannica). Most animals show separate sexes in nature. Some invertebrate animals, such as nematodes (C. elegans, C. briggsae), earthworms, slugs, and snails, display hermaphroditic qualities, exhibiting both female and male reproductive systems (Ellis, R. Lin, S. 2014). This is fairly uncommon compared to completely sexually dimorphic animals. In these animals, dimorphism is the evolutionary result of sexual selection and genomic sexual conflict. Sex chromosomes carry genes responsible for triggering developmental cascades of genetic activation that result in sexually dimorphic traits.

Sex determination establishes the specific traits associated with male and female individuals through environmental, genetic, and chromosomal factors. In mammals, primary sex determination involves the respective differentiation of male and female individuals through the development of the gonads, and is based entirely on the genetic components expressed during development (Gilbert, S.F. 2000). This process alone does not determine the complete sexual characteristics of a mammal. Secondary sex determination encompasses the external, visible, or otherwise non-gonadal phenotype of a species, often influenced by the activity of the endocrine system, and the release of hormones from either the male testes or the female ovaries (Gilbert,

2000). The process of sex determination in insects is slightly different than that of mammals. It consists entirely of genetic cascades and gene activation during embryonic development. Insects do not undergo a process synonymous with secondary sexual dimorphism (Hopkins, B. Kopp, A. 2021).

1.2: Lepidoptera

The Lepidopteran order includes the world's butterflies and moths. Consisting of around 180,000 different species, it is one of the world's most species-rich orders (Mitter, et al. 2017). The sex determination system in Lepidoptera is poorly understood. Lepidoptera operates on female heterogamy using the female ZW karyotype, as well as the male ZZ karyotype (Yang, et al. 2021). The Painted Lady butterfly, *Vanessa cardui* (Nymphalidae) is the world's most common and widely distributed species of butterfly, and is often the subject of observational and experimental studies due to its abundance in nature and short life cycle (Mitter, et al. 2017). *Vanessa cardui* have an average wingspan of between 5 and 7 centimeters, and are characterized by orange, black, and white patterning on the ventral side of their wings (Connahs, et al. 2016). They are polygynous animals, and males often mate with several females during their lifetimes. Females produce around 500 eggs in their lifespans (Gilbertson, A. 2023). *Vanessa cardui* often use multiple locations as they lay their eggs, such as certain leaves or flowers (Gilbertson, A. 2023). Overall, *Vanessa cardui* provides a stable and reliable animal model for a variety of different studies.

1.3: *Vanessa cardui* Development

From egg-laying to death, the *Vanessa cardui* lifespan is between 15 and 29 days. Eggs are typically laid on plants that attract *V. cardui*, such as hollyhocks (*Alcea* sp.). Eggs hatch within three to five days of oviposition (Johnson, S. 2019). During the larval stage, caterpillars require a consistent food and water source. They also spin silk, which assists them in remaining attached to their food source. Larvae periodically molt as they grow and develop. Average *Vanessa cardui* larvae are five centimeters long when fully grown (Johnson, S. 2019). The *Vanessa cardui* larval stage typically lasts 8-10 days (Johnson, S. 2019). In order to begin the pupal stage, larvae hang themselves upside down from a surface, and molt to form a pupa or chrysalis. This stage lasts about seven days. After this period, the adult butterfly emerges from the chrysalis (Johnson, S. 2019). Initially, adult wings are folded and crumpled. Upon eclosure, *Vanessa cardui* adults continue to hang upside down in order to let their wings inflate and dry. After this process is complete, they begin their adult life. They begin mating around 48-72 hours after eclosure (Johnson, S. 2019). Adulthood lasts between 14 and 28 days, depending on several environmental factors, such as temperature ranges and surrounding predators (Johnson, S. 2019).

There aren't many visible differences between adult males and females in *Vanessa cardui*, but they do exist. Some differences include size. Female *Vanessa cardui* butterflies are typically larger than male butterflies, although this is not always the case (Rudoy, et al. 2017). Other differences between females and males include the presence of more hair on male forelegs, as well as three rows of tarsal claws on female forelegs (Figure 1, Figure 2). Female butterflies use tarsal claws to scratch the surface of a leaf, or otherwise suitable environment when they prepare for laying eggs. *Vanessa cardui*, as well as other species of Lepidoptera, lack sex combs that are common in Drosophila. These sex combs exist in Drosophila and some other insects to

assist males in mating (Kopp, A. 2011). However, mating behavior in Lepidoptera does not require sex combs. When mating, male butterflies will typically pursue females by following them. They will attempt to point their abdomen towards the female. If the female wishes to mate, she will allow the male to attach to her, and the mating pair will be physically joined by the posterior of their abdomens for around 1-3 hours (Rutowski, R. L. 1998, Fox, R. 2013). About 24 hours after mating, females will begin to lay eggs. As stated earlier, they will find a leaf or other amenable surface for their eggs. Female butterflies will often remain near the leaf or other breeding ground for the duration of their egg laying, as the stream of eggs is continuous after they have mated. Males will mate with multiple females throughout their lifespans, typically until death (Rudoy, et al. 2017).

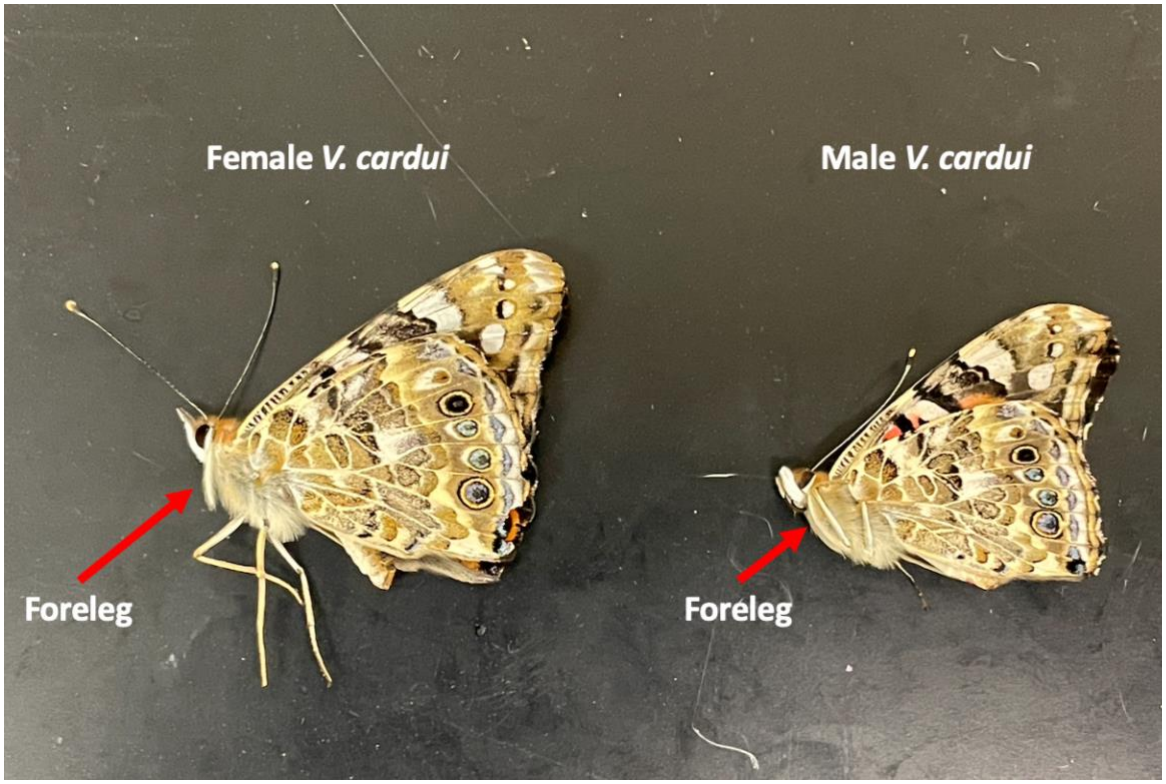

Figure 1. *Vanessa cardui* wild type female (left) and male (right) to display physical phenotype, foreleg location, and differences between sexes. *Vanessa cardui* females are typically slightly larger than males, but this is not guaranteed. There are no other sexually dimorphic differences in *Vanessa cardui* visible to the naked eye.

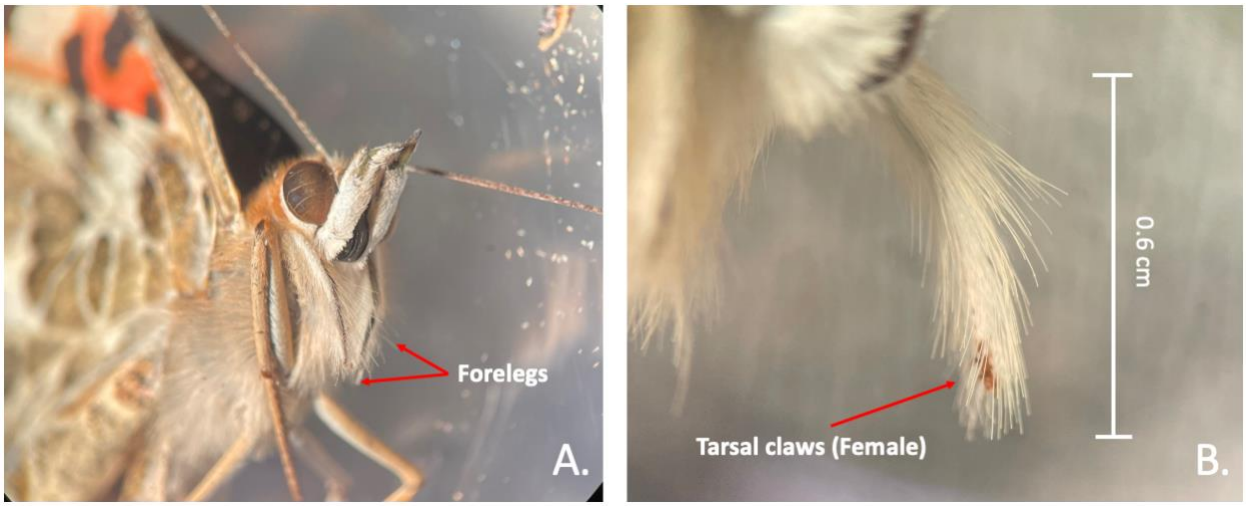

Figure 2. Microscopic views of *Vanessa cardui* and notable structures of interest. (A) *Vanessa cardui* female head, forelegs, and front legs. Image was taken at 2.5x magnification with a dissecting microscope. (B) Female *V. cardui* foreleg, with view of tarsal claws. Tarsal claws, or lack thereof, are only visible under a microscope and cannot be properly distinguished with the naked eye. Image was taken at 2.5x magnification with a dissecting microscope.

Figure 3. Microscopic view of *Vanessa cardui* vestigial forelegs. (A) Female foreleg with three rows of tarsal claws, used for scratching at surfaces of leaves and potential egg-laying grounds. (B) Male foreleg, lacking tarsal claws and containing more hair than the average female *V. cardui* foreleg. Images were taken on a dissecting microscope at 2.5x magnification.

1.4: CRISPR/Cas9 and Mosaicism

CRISPR/Cas9 is commonly used in medicinal research to edit and revise DNA sequences of live cells. CRISPR techniques are often used to generate cellular and animal models, as well as transgenic animals. The CRISPR/Cas9 process involves two components, including a short-guide RNA or sgRNA to target a specific gene of interest, as well as a Cas9 endonuclease that cleaves the phosphodiester bonds on both DNA strands, causing a double-strand break. Cas9 will typically be guided by an sgRNA to cut the gene in the appropriate places for ideal CRISPR activity. An sgRNA primer that binds to the genomic DNA (gDNA) of an organism is typically around 18-20 nucleotides long. In order to induce the double-strand break of the gDNA, a specific DNA sequence, known as a protospacer adjacent motif (PAM) must be added to the 3’

end of the sgRNA. The PAM sequence is usually between 2 and 5 nucleotides long, and reduces flexibility of the gene editing process, ensuring as much precision in the double-strand break as possible. Animal cells will typically repair the CRISPR-induced double-strand break through non-homologous end joining, which may lead to random deletions or insertions of DNA. This raises the possibility of producing frameshift mutations that render the gene of interest nonfunctional after the CRISPR process.

When performing CRISPR/Cas9-mediated gene editing on animals, specifically with the goal of knockout or transgenic individuals, it is common to observe mosaicism when making phenotypic and genotypic observations. Mosaicism is a state in which an individual has two or more genetically different sets of cells in their body (Queremel Milani, D. Chauhan, P. 2022). In the case of this experiment, an organism that has undergone CRISPR gene-editing may possess two or more cell populations that display different genotypes. When CRISPR/Cas9 components are introduced into an individual, they are typically injected as DNA, RNA, or proteins directly into an animal embryo (Mehravar, et al. 2019-2). This is done due to the sensitivity of the individual during its early development, and to ensure the highest probability of gene targeting and cleaving. During embryonic development, the CRISPR/Cas9 system may continue to cleave genes at different times, resulting in gene editing across a variety of developmental stages. In the case of *Vanessa cardui*, embryonic CRISPR injections typically target genes responsible for wing patterning, as changes in the wings are easily visible. Studies have observed the results of targeting wing pigmentation genes such as *spalt* and *DII*. Knockout *V. cardui* in these experiments show visible mosaic wing development, displaying a mixture of wild-type and mutant patterning. Genotyping of these animals confirms both the presence of wild-type *V. cardui* DNA and knockout/CRISPR-edited DNA (Mehravar, et al. 2019-2, Zhang, L. Reed, R.

2016). Overall, this experiment involved the observation and analysis of potential mosaic phenotypes. Since *V. cardui* do not have obvious sexually dimorphic qualities other than the differences in male and female forelegs, the individual forelegs of CRISPR-edited animals are of interest for observation and data collection.

1.5: *masculinizer* and Other Sex Determination Genes

Sex determination mechanisms in *Vanessa cardui* and other Lepidoptera are largely unclear. However, sex determination is fairly well understood in *Drosophila melanogaster*. Drosophila embryonic cells differentiate based on their chromosomal components and resulting sexual identity (Salz, H. Erickson, J. 2010). Drosophila have an additional aspect of their sexual development, called dosage. The dosage, or number, of X chromosomes in an animal determines its sexual identity (Krzywinska, et al. 2021). Female Drosophila have an XX karyotype, while male Drosophila have an XY karyotype. Sex determination is based on the level of transcription of X-linked genes, including *runt, sisA, scute,* and *unpaired.* Higher levels of transcription of these genes leads to the earlier activation of promoter *Sex-lethal* (*Sxl*). The early expression of *Sxl* leads to an auto-regulatory loop that results in the development of female flies (Barbash, D. Cline, T. 1995). This development is encouraged by the female isoform of the gene *doublesex* (*dsx*). *dsx* is among the most highly conserved sex determination genes across several insect groups. *dsx* encodes a transcription factor that produces two different isoforms in each sex. These isoforms, *dsxF* (female) and *dsxM* (male), act as transcriptional activators for the development of individuals into one sex (Salz, H. Erickson, J. 2010). In female Drosophila, the auto-regulatory loop of *Sxl* leads to the expression of *dsxF*, which encourages female development (Niessen, et

al. 2001). In male flies, the single X chromosome does not encourage the level of transcription that occurs in female flies. The resulting *Sxl* isoform is unable to produce a functional protein, and allows for the persistence of *dsxM*, which encourages male development (Niessen, et al. 2001, Salz, H. Erickson, J. 2010).

The Lepidopteran model of sex determination is less conserved, and differs highly from Drosophila. Lepidopteran individuals possess either a ZZ (male) or ZW (female) karyotype. This deviates from the X0/XX chromosomal system in Drosophila, as Lepidoptera do not rely solely on the dosage of certain genes in order to differentiate between female and male insects (Yang, et al. 2021). Examinations of the silkmoth *Bombyx mori* observed that the female W chromosome contains a locus called *feminizer (fem).* This locus, so far only known from *B. mori*, is responsible for producing a PIWI-interacting RNA, or piRNA, that assists in female development during the embryonic stage (Yang, et al. 2021, Katsuma, et al. 2019). In female lepidoptera, *fem* piRNAs degrade mRNAs released from *masc*, suppressing the development of male Lepidoptera sex characteristics (Krzywinska, et al. 2021, Visser, et al. 2021). *masc*, while present on the Z chromosome in both sexes, will only persist in males. The absence of a W chromosome and *fem* piRNA results in the persistence of *masc,* and ultimately male development through the male isoform of *doublesex*, dsxM. (Katsuma, et al. 2019). The persistence of the masc gene also controls dosage compensation in addition to the development of masculine traits. Dosage compensation is a mechanism that regulates X or Z linked gene expression with an animal's autosomes (Katsuma, et al. 2019). Drosophila utilize dosage compensation via hyper-transcription of their hemizygous X chromosome. Lepidopterans, on the other hand, require *masc* to induce dosage compensation. Early embryonic knockouts of *masc* using silencing RNA (siRNA) injections resulted in the hyper-upregulation of Z-linked genes in male *B. mori*, which

ultimately led to male embryonic death. However, female *B. mori* with this knockout were unaffected, instead containing the typical female *dsx* isoform *dsxF mori* (Katsuma, et al. 2019). These findings indicate that successful *masc* knockouts have the capability to influence gene expression and sex determination during development. Another gene that is thought to be involved in male Lepidoptera development is *P-element Somatic Inhibitor* (*PSI*). *PSI* is highly conserved across several lepidopteran groups. It is required for male Lepidopteran development, and plays a role in encouraging the splicing of *dsxM* in male butterflies (Yang, et al. 2021, Suzuki, et al. 2008).

In addition to *B. mori*, *masc* also initiates development in the flour moth, *Ephestia kuehniella* (Pyralidae). These animals contain a conserved *masc* ortholog, EkMasc, on the Z chromosome. This ortholog encodes a *masc* protein that, similar to *B. mori*, persists only in male embryos. Knockouts of this gene in *E. kuehniella* embryos resulted in overwhelming shifts in favor of female development and presentation of the female *doublesex* (*Ekdsx*) isoform (Visser, et al. 2021). This suggests that the role of *masc* as a regulator of masculinization and dosage compensation is conserved in *E. kuehniella*. These findings suggest that *masc* could have a conserved role across other Lepidopteran species, including *Vanessa cardui*.

1.6: Hypothesis and Motivation

Given the documented role of *masc* in sex determination in *Bombyx mori* (Bombycidae) and *Ephestia kuehniella* (Pyralidae), it is possible that this gene plays an ancestral and conserved role specifying male development across Lepidoptera. To test the role of *masc* in the genetic control of sexual dimorphism more broadly in Lepidoptera, I created mutations in the

masculinizer gene, and examined its effects on the sex characteristics of *Vanessa cardui* (Nymphalidae). Using CRISPR/Cas9 gene editing, I observed the effects of *masc* knockouts on animal survival during embryonic, larval, and pupal development, as well as adult anatomy, behavior, and mating capabilities. I used genome editing methods similar to Zhang, et al. (2017) to produce mosaic individuals. Specifically, I performed microinjections using glass capillary needles on *V. cardui* embryos, and closely observed their development in the time following the process. Suspected knockouts were observed through examination of animal forelegs and presence or absence of tarsal claws. Data collection involved the thorough comparison of sexually dimorphic phenotypes between a control and treatment group of *V. cardui*. I tested individuals for fertility in mating experiments and analyzed changes in mating behavior using a series of 72-hour behavioral assays. I was motivated to perform this research because of my interest in CRISPR/Cas9-based research, morphometry, and molecular biology.

CHAPTER TWO

MATERIALS AND METHODS

2.1: Butterfly Husbandry

Throughout the experiment, *Vanessa cardui* were kept in captivity and allowed to reproduce freely to ensure the presence of healthy adults that could produce viable eggs for microinjection. Upon receiving a new *V. cardui* generation, individual larvae were separated into 37-ml plastic cups (Frontier Agriculture Sciences, Delaware, USA), along with a half-tablespoon of artificial larval food (Southland Products Inc., Arkansas, USA). Each cup was covered with a Kimwipe layer, which provided a rough surface on which larvae could effectively pupate, and a plastic lid to discourage the evaporation of moisture from the food.

It is common among researchers working with *Vanessa cardui* to observe an unidentified pathogen in late-stage larvae before pupation. This pathogen typically presents with mold in larval fecal matter and failure to thrive. Infected pupae often do not eclose, resulting in a poor generation of animals (Myers, J. Cory, J. 2015). All infected larvae were euthanized by placing them in a freezer at -20ºC overnight, and disposing of them as well as the plastic cup they were enclosed in. Pupae were pinned upside down from the KimWipe in a Styrofoam box to ensure proper moisture for development. After eclosure, adults were transferred to a netted enclosure and left to socialize. A hollyhock (*Alcea* sp.) plant was placed inside the enclosure 48 hours after all adult butterflies had moved from the pupation chamber. Adults were left to lay eggs on the hollyhock leaves. Eggs were collected from the leaves to establish future generations.

2.2: sgRNA Construction

To design the short guide RNA models for the CRISPR/Cas9 gene editing, Geneious™ software (BioMatters Inc., Auckland, New Zealand) was used to isolate specific sequences of interest for *masc*. A candidate gene in *V. cardui* was obtained starting from a known *masc* sequence from *Bombyx mori.* Sequences were then compared using a Basic Local Alignment Search Tool (BLAST) to compare the *B. mori* selected sequence to the *V. cardui* transcriptome. This process produced a *V. cardui* gene transcript with a similar sequence to that for *B. mori* (Appendix 1). The resulting *V. cardui* sequence was transferred into Geneious™, and annotated with potential sgRNA target sequences containing predicted effective CRISPR sites. Ultimately, two *masc* sgRNA sequences were chosen (Table 1). These two sgRNAs were found by observing the optimal CRISPR sites found in Geneious™, and choosing ones based on the suggestions discussed in Zhang & Reed (2017). These target sites needed to be at a reasonable distance before the start (Met) and stop codon of the gene. This was done to ensure the creation of a non-functional *masc* allele with successful CRISPR activity. Geneious™ color-coded all possible primers, displaying suitable ones in green. The position of each of the two masc sgRNAs were noted (Table 1). The first sgRNA was 24 base pairs long. It was on the reverse sequence and had endpoints 307 → 284 (5′ → 3′). This sgRNA was labeled "*masc*-284." The second sgRNA was also 24 base pairs long. It was on the forward strand and had endpoints 526 → 549 (5′ → 3′). This sgRNA was labeled "*masc*-526." Both primers were subsequently used in the synthesis process of the sgRNAs.

For *in vitro* transcription of sgRNAs, all guide sequences were fitted with a 5-prime T7-RNA polymerase promoter sequence, and a 3-prime Cas9 recognition sequence. For each target sequence, a gene-specific forward primer was designed based on the sgRNA sequence. The other

primer was a universal sequence containing only the Cas9 recognition sequence. From these sgRNA primers, a template-free PCR was performed in order to produce a double-stranded DNA sequence with a promoter, as well as the necessary sequence for the *masc* target and Cas9-binding sequence.

2.3: sgRNA Preparation: PCR

In order to prepare Cas9/sgRNA solutions for microinjections, several preparatory procedures were performed, similar to those described in Zhang & Reed (2017). First, a series of PCR reactions were performed using gene-specific primers, universal-Cas9sg-R primer, and JumpStart Taq Ready-mix enzyme solution (Sigma-Aldrich, Massachusetts, USA). Each *masc* sgRNA (*masc*-284 and *masc*-526) underwent its own PCR reaction. The PCR reactions were assembled using 5.5 µl of nuclease-free water, 0.5 µl gene-specific masc primer, 0.5 µl universal-Cas9sg-R primer, and 6.0 µl JumpStart Taq Ready-mix solution. The reactions were mixed and placed in a thermocycler. The thermocycler program was as follows:

98ºC	10 seconds	|
60ºC	30 seconds	| 35 Cycles
72ºC	15 seconds	|
72ºC	10 minutes	
12ºC	Infinite hold	

The PCR reactions were removed from the thermocycler within 18 hours of the program's completion, and stored at -20ºC until further use.

2.4: sgRNA Preparation: RNA Synthesis

The PCR products were used as templates for *in vitro* RNA synthesis. Double-stranded RNA (dsRNA) for the *masc* oligos was transcribed using T7 RNA polymerase and a MEGAscript T7 Transcription Kit (Thermo-Fisher Scientific, Massachusetts, USA). This procedure uses elements from Laslo et al. 2022. First, two 20 µl RNA synthesis reactions were assembled for each of the two masc PCR products in 200 µl PCR tubes. These reactions included 7 µl of nuclease-free water, 8 µl total (2 µl each) of NTPs (A, C, T, G), 2 µl of reaction buffer (10X), 1 µl template *masc* PCR product. The reactions were mixed using a pipette, and incubated at 37ºC for 4 hours. The reactions were removed from the thermocycler, and 1 µl of TURBO DNase (Thermo-Fisher Scientific, Massachusetts, USA) was added to each reaction. The reactions were placed back into the thermocycler and incubated at 37ºC for 15 minutes. After the incubation was finished, the reactions underwent the protocol "Anneal dsRNA." This protocol inactivated the DNase enzyme through heat, and allowed the reactions to cool properly to ensure a stable secondary structure of the resulting complementary RNA. The program was as follows:

95ºC	3 minutes
-0.1ºC/s ramp	Approximately 9 minutes
45ºC	1 minute
12ºC	Infinite hold

The reactions were then removed from the thermocycler, and 79 µl of nuclease-free water was added to each reaction tube. The mixtures were then transferred to nuclease-free 0.5 µl tubes, and stored at -80ºC until further use.

2.5: sgRNA Preparation: RNA Purification

The *masc* dsRNA underwent RNA purification to remove impurities and prepare for microinjection into *V. cardui* embryos. This procedure was performed on ice. The RNA samples were first precipitated by adding 50 µl of 7.5M ammonium acetate and 300 µl of 100% ethanol, both of which were both stored at -20ºC until use. The samples were vortexed, and left to precipitate for around 1 hour at -20ºC. The samples were then centrifuged at 12,000 rpm for 20 minutes at 4ºC. All supernatants were discarded from both samples using a micropipette. The pellets were washed with 0.5 ml cold 70% ethanol, and centrifuged at 10,000 rpm for 5 minutes at 4ºC. The supernatants were discarded using a micropipette. The pellets were then dried in a vacuum centrifuge for 15 minutes, or until the pellets began to appear opaque and white. The caps of each reaction were left open to ensure evaporation. The pellets were then resuspended in 30 µl of nuclease-free low Tris-EDTA buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). The mixtures were observed using a spectrophotometer to determine their respective concentrations and purities. The quality of the RNA was determined by running the samples on a 2% agarose gel. If the masc sgRNA products were found to be of optimal quality, they were stored in 0.5 ml tubes at 80ºC until microinjection.

2.6: *in vitro* Digestion with sgRNA

Prior to the microinjection procedure, the *masc* sgRNAs were observed using an *in vitro* digestion assay. This procedure utilized several methods found in Mehravar, et al. (2019-1), to verify the efficiency of the designed sgRNAs for CRISPR cleavage of *V. cardui* gDNA. Ultimately, this procedure determined the capability of the masc primers for a successful knockout into *V. cardui* DNA. First, two PCR reactions were assembled using 7.5 µl nuclease-

free water, 2.5 µl magnesium chloride, 2.0 µl gDNA, 1 µl forward primer, 1 µl reverse primer, and 12.0 µl JumpStart Taq Ready-mix. Magnesium Chloride was added to the reaction in order to enhance the enzymatic activity of the polymerase, ultimately increasing the amplification of the DNA. Template DNA for the PCR consisted of genomic DNA (gDNA) collected via DNA extraction using a Qiagen® DNeasy kit (Qiagen Inc. Mississauga, Ontario, Canada). The reaction was mixed with a micropipette and placed into the thermocycler. The program used for these reactions was as follows:

98ºC	5 minutes	
98ºC	10 seconds	|
48ºC	30 seconds	| 45 cycles
72ºC	90 seconds	|
72ºC	5 minutes	
12ºC	Infinite hold	

The PCR products were then used as components of the *in vitro* digest reactions, which were assembled in separate 0.5 ml tubes. Each digest was assembled on ice, and contained 13 µl nuclease-free water, 3 µl NEBuffer 3.1, 1 µl purified *masc* sgRNA (1 ug/ µl), 3 µl Cas9-NLS (200 ng/ µl), and 10 µl *masc* PCR product (either *masc*-284 or *masc*-526). The digests were mixed thoroughly with a micropipette, and incubated at 37ºC for two hours. The products were removed, and 1 µl Proteinase K was added to each. This was done to destroy the Cas9-NLS in each reaction, and release the digested DNA. The reactions were placed back into the incubator for 10 minutes at 65ºC. Proteinase K is resistant to heat up to around 65ºC. The products were then stored at -80ºC in preparation for gel electrophoresis. The *masc* PCR product, as well as the

digested product, underwent gel electrophoresis alongside a 1500 bp ladder, and significant DNA fragments were measured and recorded.

2.7: Cas9 Microinjection Solution Preparation

Microinjection solutions were prepared in order to produce *in vivo masc* knockouts in *V. cardui* gDNA. The previous *in vitro* assay involved the simple splicing of the *masc* target sequences. The *in vivo* microinjection procedure also included the natural DNA repair mechanisms (non-homologous end-joining) of the animals. Prior to microinjection, 5 µl aliquots of Cas9-NLS in 0.5 ml tubes were thawed over ice. Cas9-NLS contains a nucleic localization signal on both its N and C terminus that assists in inducing double-strand breaks of DNA (Hu, et al. 2018). Cas9 stock was made by mixing 34.3 µl of nuclease free water and 9.8 µl of phenol red (0.5% solution) in a single Cas9-NLS aliquot. After mixing, 5 µl aliquots of Cas9 stock were stored at 80ºC in 0.5 ml tubes. To prepare the microinjection solution, 5 µl of *masc* sgRNA primer (*masc*-284 or *masc*-526) was added to a 5 µl aliquot of Cas9-NLS stock, along with 10 µl of nuclease-free water. The final concentrations of the solution components were as follows: 250 ng/µl Cas9-NLS, 125 ng/µl sgRNA, and 0.02% phenol red. The solutions were mixed with a micropipette and incubated at room temperature for 10 minutes. The solutions were then stored on ice until microinjection.

2.8: Microinjection

Microinjection needles were pulled from borosilicate glass capillary tubes using a Sutter Instruments P-97 Micropipette Puller with parameters as follows: Ramp = 699, Pull = 77, Vel = 16, T = 60. Needles were stored in a large petri dish with two rows of clay to immobilize the

needles. Prior to the microinjection procedure, *Vanessa cardui* embryos were collected between 3-5 hours after laying. A plastic cup was prepared with a small amount of larval food, and a plastic lid was prepared with a piece of double-sided tape on its underside. Eggs were placed in groups of either 15 or 20 on the tape, and stored in the plastic cup until the microinjection solution was fully prepared on ice. 1.5 µl of the microinjection solution was back-loaded into the needles using a 2 µl pipette, and the needle was subsequently connected to a needle holder. Pressure was provided by a small air compressor equipped with a low-pressure regulator. Eggs were placed under a stereomicroscope, and injected one at a time with a pressure of 4 psi. The eggs were separated into 10 groups of 15 (day 1) or 10 groups of 20 (days 2 and 3).

Day	Number injected: *masc*-284	Number injected: *masc*-526
1	75	75
2	100	100
3	100	100

Half of each group was injected with *masc*-284, while the other half was injected with *masc*-526. Injected embryos were placed in an incubator at 28ºC to develop.

2.9: Microinjected Animal Husbandry

After microinjection, groups of eggs were stored in labeled 37 ml plastic cups (Frontier Agriculture Sciences, Delaware, USA), and kept in an incubator at 28ºC with an 8/16 hour light/dark cycle. Animals were kept separate based on which sgRNA primer they were injected with. Eggs were observed daily inside the incubator. Deceased eggs were collected daily and stored in a petri dish at -80ºC. Surviving larvae were kept in the 28ºC incubator, and observed daily. Larval food was occasionally changed, and larvae were moved into individual cups as they

continued to develop. Deceased larvae were collected daily and stored in a petri dish at -80ºC. Surviving pupae were removed from the 28ºC incubator and placed in a styrofoam pupation chamber at room temperature. The interior of the pupation chamber was sprayed with nuclease-free water daily. Upon eclosure, adults were carefully transferred to individual 3.7 liter insect terrariums, along with food source and a sticky note sheet on the terrarium's wall in order to hang vertically. Adult butterflies were isolated for 48 hours in the terrariums to prevent any potential mating or interactions with other injected butterflies that would compromise the results of the subsequent behavioral mating assay.

2.10: Behavioral Mating Assay

Over the 48 hour isolation period, all injected adults were observed to determine anatomical differences and changes in phenotype. To observe impacts of *masc* mosaic knockouts on mating behavior, a 72 hour study was conducted isolating adult *V. cardui* that were suspected to contain successful CRISPR/Cas9 knockdowns. Upon eclosing, adult butterflies were separated into 3.7 liter insect terrariums, and left isolated for 48 hours. They were then placed into an incubator at 28ºC with an 8/16 hour light/dark cycle and socialized with a wild type *V. cardui* butterfly. Pairs were left to mate over a 72 hour period, and were observed three times each day for a period of 30 minutes: once at 11:00 am, once at 2:00 pm, and once at 4:00 pm. Any mating behavior, or lack thereof, was recorded.

2.11: Anatomical Observations and Photomicrography

After the behavioral mating assay, injected animals were placed in netted enclosures. *masc*-284 injected butterflies were placed separately from *masc*-526 injected butterflies. Since

Vanessa cardui forelegs have easily visible sexually dimorphic traits (tarsal claws in females,) the individual forelegs of each animal were observed. Each foreleg was removed from the animals and stored separately in 1 ml centrifuge tubes marked 1-40. For imaging, each *V. cardui* foreleg was placed on a petri dish next to a 5 cm ruler. A dissecting microscope was used along with a Motic® Moticam 10+ photomicrography camera and Motic Images Plus 3.0 software to obtain images of each individual foreleg within the injected group. Animals were imaged at 2.5x magnification alongside a 4 cm ruler for scale.

2.12: Statistical Analysis

During the process of development for the microinjected animals, the date of death, as well as the developmental stage at time of death, was recorded for each animal in a Microsoft Excel spreadsheet. An uninjected negative control group was constructed using a generation of 300 eggs, and the animals were observed and counted as they progressed through developmental stages to adulthood. After data collection was complete, analysis of stage-based animal survival was done with RStudio. Necessary packages included tidyverse, survival, ggsurvfit, gtsummary, tidycmprsk, condsurv, survminer, ggpubr, and viridis. To visualize survival curves between the control and experimental groups, Kaplan-Meier curves were constructed. A Kaplan-Meier curve presents the survival probability of a set of individuals over a given period of time (Goel, et al. 2010). This time period is broken up into individual intervals, or "bins." In the case of this experiment, Kaplan-Meier curves were helpful for depicting survival probabilities during the transitions from injection to hatching, hatching to pupation, and pupation to eclosure. Chi-squared tests were run between each sgRNA-injected group and the negative control to determine any respective significant differences in survival.

CHAPTER THREE

RESULTS

3.1: sgRNA Construction

A target sequence for the *masculinizer* gene in *V. cardui* was obtained by performing a BLAST search using an existing *B. mori masc* gene sequence (Appendix). The *V. cardui* transcript was uploaded to Geneious™, and sgRNA target sites were observed. Two *masc* sgRNA sequences of best fit were chosen (Table 1). The sequences were produced *in vitro* via flanking by a 5' T7 RNA polymerase and a Cas9 recognition sequence (Table 1). PCR reactions were performed using the two *masc* sgRNAs as gene-specific primers, as well as a universal primer. The reactions were observed using gel electrophoresis, displaying products with a length of 122 bp (Figure 4). The PCR products were used as templates for *in vitro* RNA synthesis to produce the final *masc* sgRNA primers. RNA yields and purities were recorded using a spectrophotometer. The *masc* sgRNA with the 5' position of 284 (*masc*-284) had an RNA yield of 5,311.1 ng/ μl, and an A260/A280 of 1.36. The masc sgRNA with the 5' position of 526 (*masc*-526) had an RNA yield of 4,171.1 ng/ μl, and an A260/A280 of 2.08 (Table 2).

Gene	Position	Strand	Primer Name	Primer Sequence
masc	284	Reverse	7-Vc-masc-284-Cas9sg	**taatacgactcactataggg**TTGTCTAGCGAAGGCGGTGG**ttttagagctagaaatagc**
masc	526	Forward	7-Vc-masc-526-Cas9sg	**taatacgactcactataggg**AGAGTCTCTTTTTTGCCACA**ttttagagctagaaatagc**

Table 1. masc sgRNA primer sequences and information obtained from Geneious™ and sgRNA oligo selection process. Bold text indicates 5' T7 RNA polymerase (front) and Cas9 recognition sequence (back).

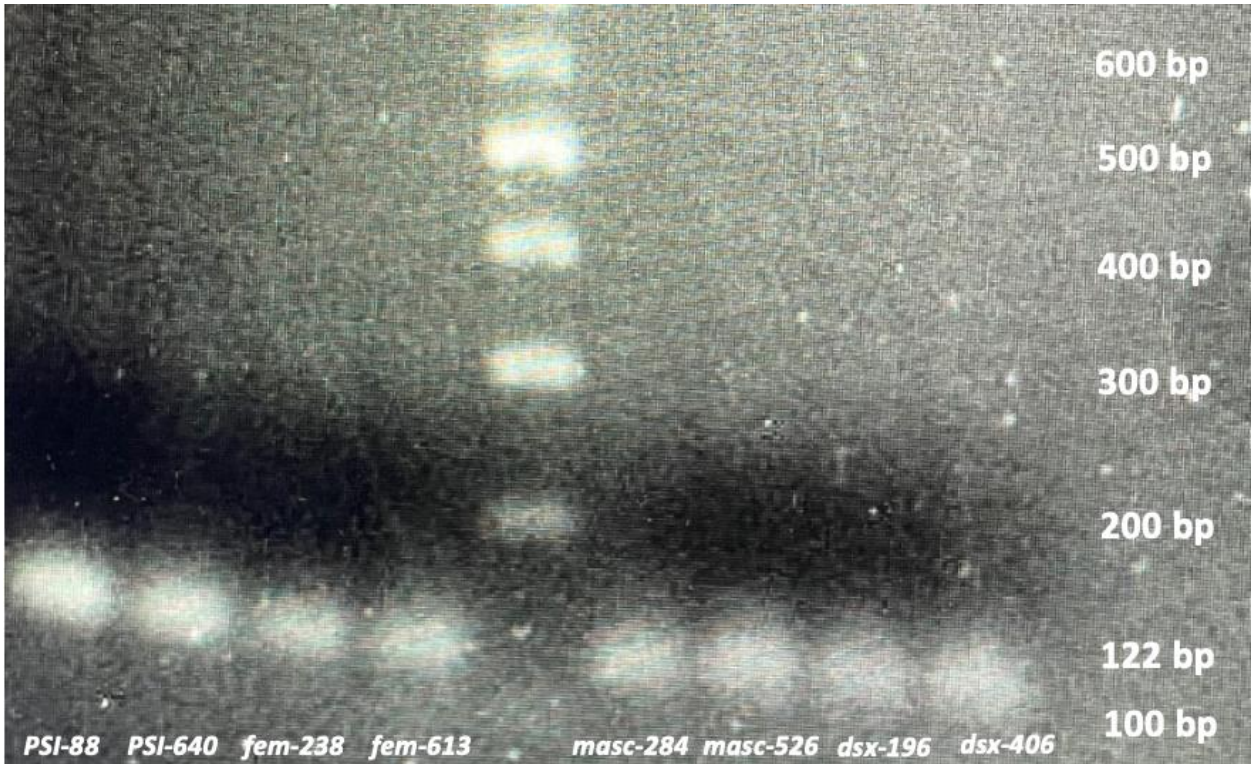

Figure 4. Gel electrophoresis of completed PCR product for two *masc* primers (284 and 526) as well as for other primers targeting sex determination genes *dsx*, *fem*, and *PSI*. All products were expected to be around 122 bp in length, due to the resulting formation of the gene-specific primer and universal primer in each reaction. Gel was measured with a 1500 bp ladder.

sgRNA Name	Concentration (ng/µl)	A260/A280
PSI-88	5,041.9	1.76
PSI-640	5,190.3	1.61
fem-238	5,140.5	1.22
fem-613	4,821.1	1.79
masc-284	5,311.1	1.36
masc-526	4,171.3	2.08
dsx-196	2,198.9	2.05
dsx-406	4,133.9	1.98

Table 2. Spectrophotometer recordings of purified RNA for *doublesex*, *feminizer*, *masculinizer*, and *PSI* sgRNA solutions prior to microinjection solution prep.

3.2: *in vitro* Digestion of DNA with *masc* sgRNA/Cas9

The results of the *in vitro* digestion assay with Cas9-NLS reveal the successful capability of the *masc*-526 sgRNA primer to assist the Cas9-NLS in cleaving the *Vanessa cardui* gDNA. Geneious™ provided a map of two potential *masc* PCR products for the digest (Figure 5A). The short PCR product (258 bp) contains a target site for the *masc*-526 sgRNA primer. It does not contain a target site for the *masc*-284 primer (Figure 5A). During the first attempt of the *in vitro* digest, it was noted that the digested PCR products remained stuck in the gel wells (See Appendix). This indicated that the Cas9 was unable to release the fragments (Mehravar, et al. 2019-1). Proteinase K was added during the digest to ensure the digestion of the Cas9-NLS, and release the digested fragments.

The *masc* PCR product treated with the *masc*-526 sgRNA and Cas9-NLS split into two distinct gene fragments of 190 bp and 60 bp, which are visible along with the undigested PCR product of approximately 250 bp (Figure 5B). The *masc*-284 sgRNA primer did not show successful digestion capability with Cas9-NLS, as there are no distinct fragments seen on the gel. There is only one fragment of approximately 250 bp, indicating the presence of the undigested PCR product (Figure 5B).

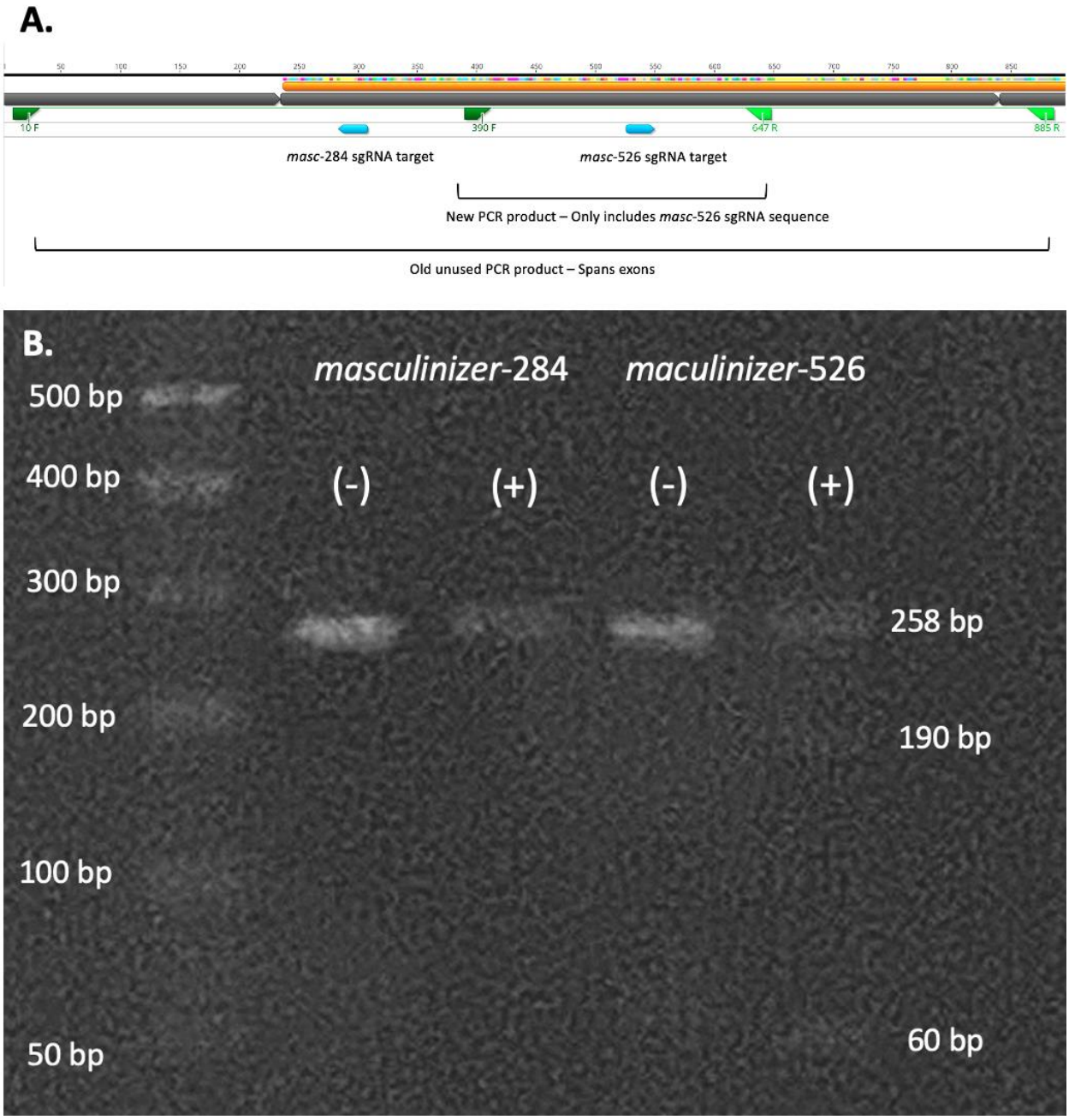

Figure 5. Predictions and results from *in vitro* Digestion of DNA with Cas9-NLS. (A) Geneious™ map displaying the PCR product used for the *in vitro* assay, and the location of both *masc* sgRNA target sites along the *V. cardui* genome. (B) Gel electrophoresis depicting *masc* PCR product before and after *in vitro* digestion with either *masc*-284 or *masc*-526, and Cas9-NLS. Undigested PCR product indicated with a (-). PCR product post-digest indicated with a (+).

3.3: Injected Egg Survival Across All Developmental Stages

A total of 550 *Vanessa cardui* eggs were injected with one of two *masculinizer* sgRNAs. The first sgRNA, *masc*-284, had endpoints 307 → 284 (5′ → 3′). The second sgRNA, *masc*-526, had endpoints 526 → 549 (5′ → 3′). 275 *V. cardui* eggs were injected with the *masc*-284 guide RNA, and 275 were injected with the *masc*-526 guide RNA. Figure 7 displays an overall visualizer of the collected survival probability data between the three measured groups. The light green represents the embryonic/egg stage, The turquoise section represents the larval stage, The blue section represents the pupal stage, and the indigo section represents the adult stage. Eggs were stored in an incubator at 28ºC with an 8/16 light/dark cycle after microinjection. Of the 550 injected eggs, 18 *masc*-284 eggs, and 17 *masc*-526 eggs hatched and became larvae. 13 *masc*-284 larvae, and 13 *masc*-526 larvae pupated successfully. 9 *masc*-284 eggs, and 11 *masc*-526 eggs survived to adulthood. A negative control group was observed using an uninjected cohort of *Vanessa cardui*. This group included 306 eggs. 66 wild-type eggs hatched and became larvae. 50 wild-type larvae pupated. 46 wild-type pupae survived to adulthood (Figure 7).

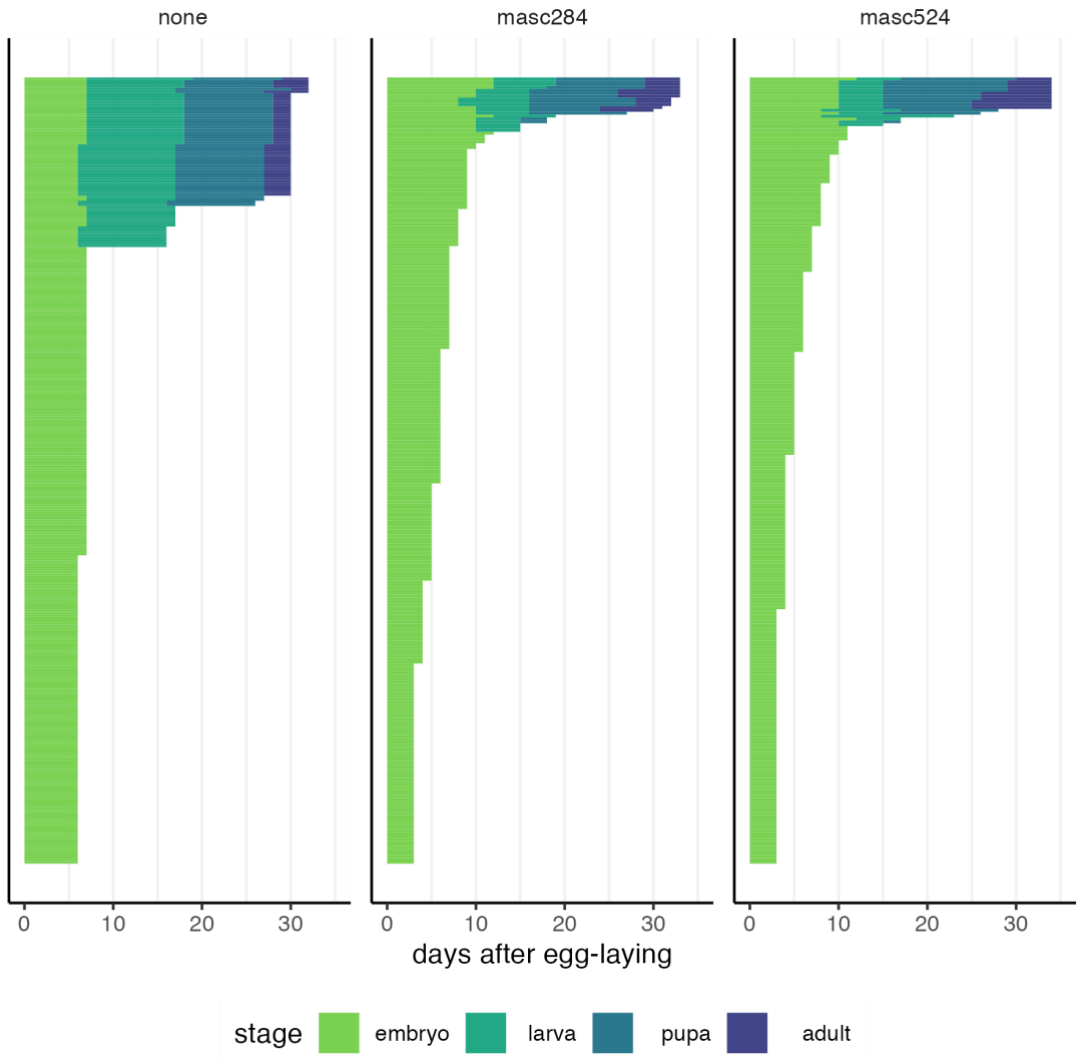

Figure 7. Developmental stage-based survival chart of microinjected adults with *masc* sgRNA primers *masc*-284 and *masc*-526 and negative control uninjected group. Wild-type larvae and pupae were reared in the laboratory at room temperature. Injected larvae were kept in an incubator at 28ºC. Injected pupae were reared in the laboratory at room temperature. Injected and wild-type Adults were stored at room temperature in netted enclosures or individual insect terrariums.

3.4: Injected Egg Survival: Embryonic Stage to Larval Stage

The survival probability of the animals was measured within each developmental stage. For injected *Vanessa cardui*, data collection began on the date of injection, and continued until all embryos had either perished or survived to the larval stage. For the uninjected control eggs, data collection began on the tentative day that each egg was laid. Eggs for the uninjected control were kept on a laboratory bench at room temperature with an 8/16 hour light/dark cycle, while injected eggs were kept in an incubator at 28ºC. This protocol difference occurred due to the time of year in which each respective generation was reared. Uninjected eggs were reared in October, and were exposed to warmer and more humid conditions. Injected eggs were reared in January, and were exposed to much colder and drier conditions.

The uninjected group of eggs maintained a survival probability of 1.00 until day 6. They then began to display a drop in survival probability to approximately 0.20 by the end of the embryonic data collection period. Ultimately, 20% of the uninjected control *V. cardui* embryos (66 individuals) survived to the larval stage (Figure 8).

The survival probability of the *masc*-284 injected group dropped from 1.00 to 0.75 by day 3. It continued to drop in increments of 0.05-0.10 per day. By day 12, all *masc*-284 embryos had either transitioned to the larval stage, or perished naturally. The final survival probability for the *masc*-284 group was 8%, indicating 18 surviving individuals (Figure 8).

The survival probability of the *masc*-526 injected group behaved similarly to the *masc*-284 injected group. It dropped from 1.00 to 0.70 by day 3, and decreased in increments of 0.05-0.15 in the next several days. By day 12, all *masc*-526 embryos had either transitioned to the larval stage or perished. The final survival probability for the *masc*-526 injected group was 9%, indicating 17 surviving individuals (Figure 8).

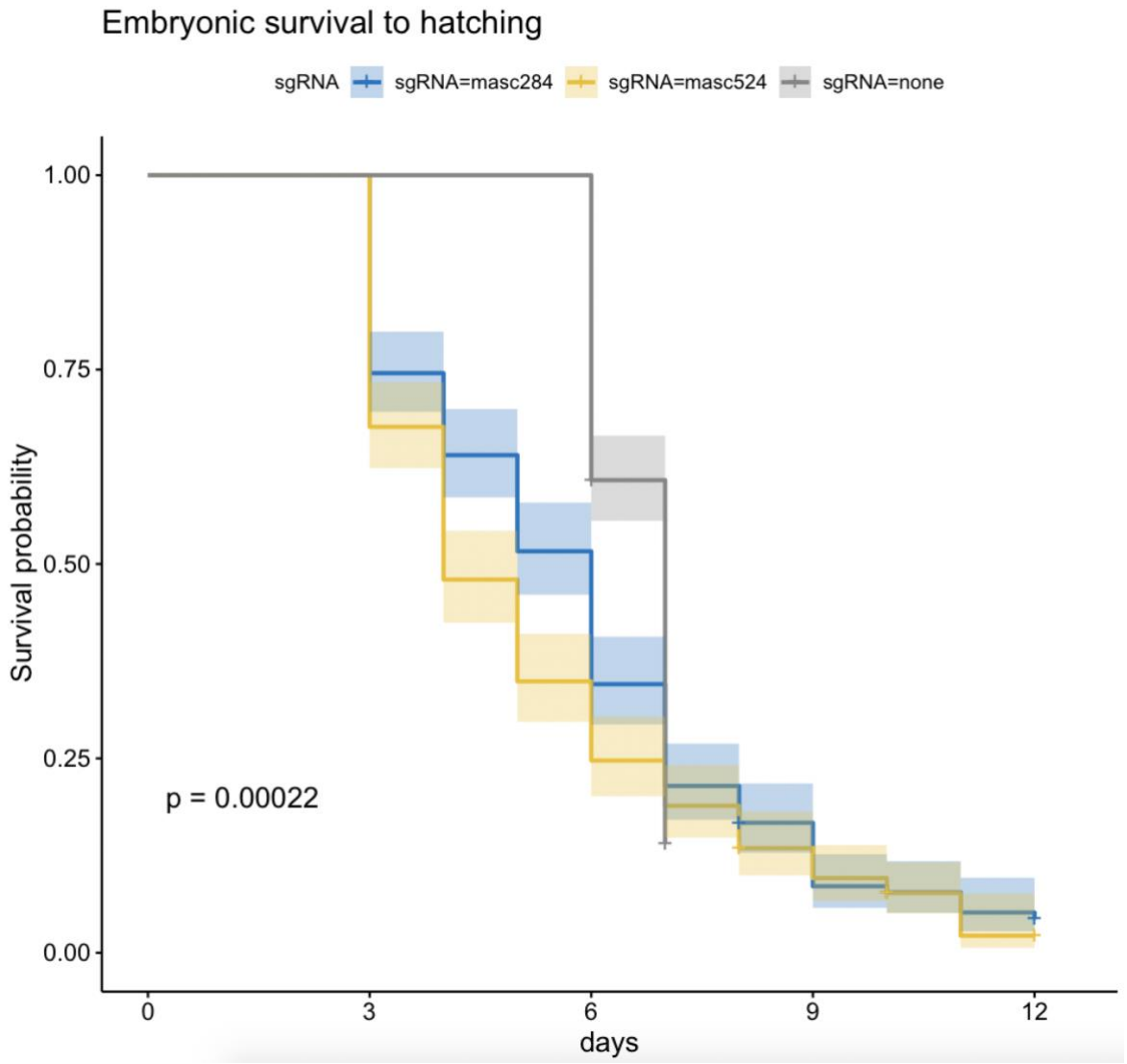

Figure 8. Developmental stage-based survival probability chart from day/time of injection to larval stage transition.

masc-284 vs. control - Embryo to Larvae	X squared = 17.765, df = 1, p = 2.499^{-5}
masc-526 vs. control - Embryo to Larvae	X squared = 20.176, df = 1, p = 7.064^{-6}

Table 3. Chi-squared analysis of embryonic to larval stage survival between *masc*-284 injected *V. cardui* and uninjected control group.

3.5: Injected Egg Survival: Larval Stage to Pupal Stage

In the uninjected group, 66 *V. cardui* larvae were alive at the beginning of data collection. The survival probability of the uninjected group remained at a value of 1.00 until day 10 (Figure 9). During this time period, second and third instar larvae began to perish due to failure to thrive or pathogen infection. The final survival probability for the uninjected group was approximately 72%. 44 larvae from the uninjected group survived to become pupae, and were subsequently moved into a styrofoam pupation chamber at room temperature. In the injected groups, there were 19 *masc*-284 surviving larvae. By day 5, the survival probability of the *masc*-284 group dropped to 0.8. By day 6, the probability had decreased to 0.74. 13 larvae in the *masc*-284 group pupated. The final survival probability in this stage for the *masc*-284 injected group was 74%. There were 17 *masc*-526 surviving larvae at the start of data collection (Figure 9). By day 5, the survival probability decreased to 0.85, and remained 0.85 for the remainder of the data collection period. 13 *masc*-526 larvae pupated successfully. One outlier larva did not pupate, instead remaining in the larval stage for all 15 days of the data collection period (Figure 9). Eventually, this individual perished without pupating.

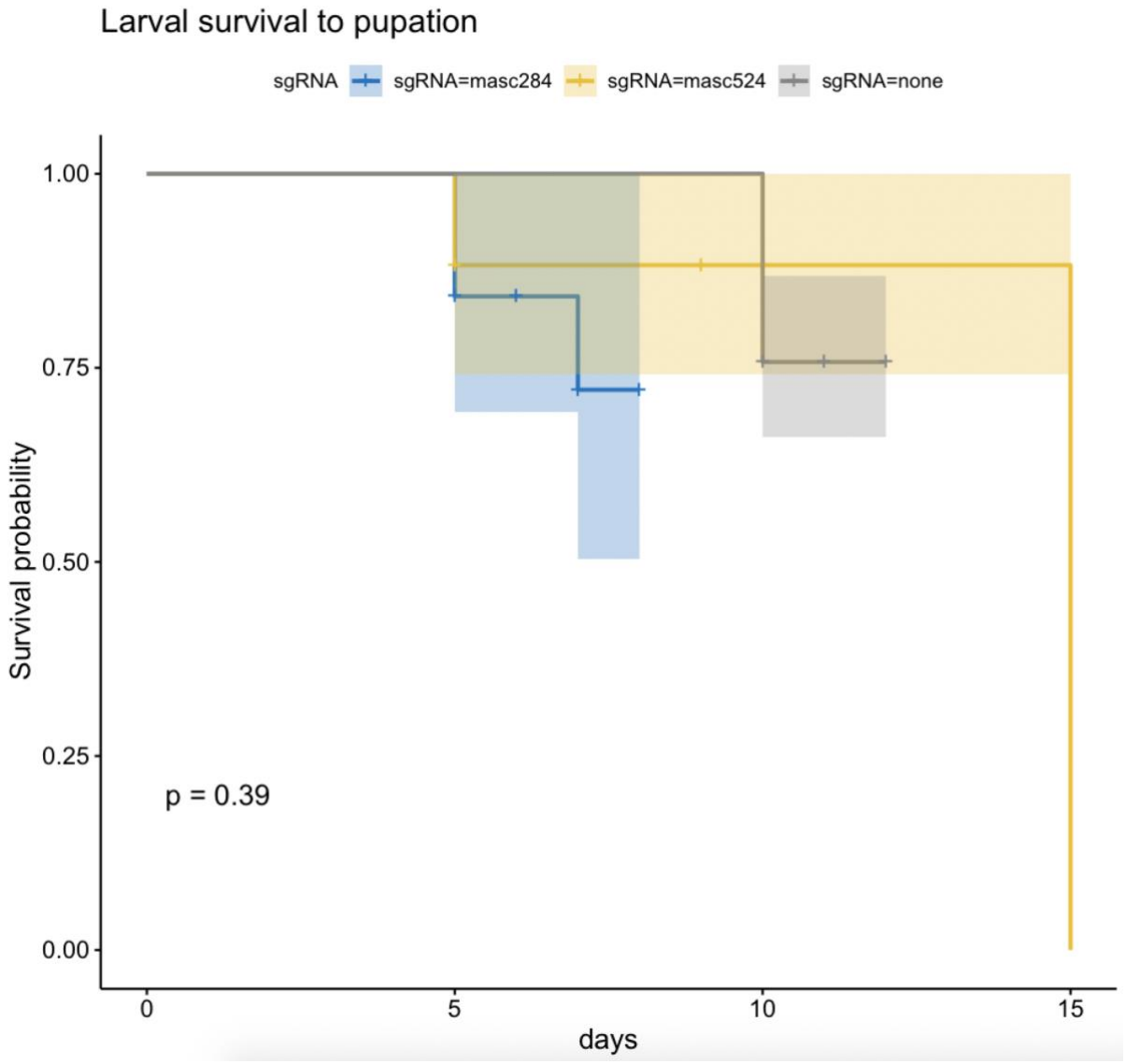

Figure 9. Developmental stage-based survival probability chart from first recorded day of pupal stage to adult stage transition. Pupae kept at room temperature for all groups.

masc-284 vs. control - Embryo to Pupae	X-squared = 12.939, df = 1, p-value = 0.0003218
masc-526 vs. control - Embryo to Pupae	X-squared = 14.096, df = 1, p-value = 0.0001738

Table 4. Chi-squared analysis of embryonic to larval stage survival between *masc*-284 injected *V. cardui* and uninjected control group.

3.6: Injected Egg Survival: Pupal Stage to Adult Stage

In the uninjected group, 50 wild-type *V. cardui* pupae entered the pupation chamber at the beginning of the data collection period. The survival probability of the uninjected group remained at a value of 1.00 until day 11 (Figure 10). During this time period, four pupae either failed to eclose, or never attempted to eclose. On day 11, the survival probability of the uninjected group decreased to 0.90, and remained 0.90 for the remainder of the data collection period. 40 adult wild-type *V. cardui* emerged and were transferred into a netted enclosure in the laboratory at room temperature. The final survival probability for the uninjected group was approximately 90% (Figure 10). In the *masc*-284 injected group, there were 13 *masc*-284 surviving pupae. By day 2, the survival probability of this group dropped to 0.85. By day 11, the survival probability of the *masc*-284 injected group continued to decrease to 0.70. This was due to several pupae dying without eclosing (Figure 10). By the end of the data collection period, there were 9 surviving *masc*-284 adult *V. cardui.* In the *masc*-526 injected group, there were 13 *masc*-526 surviving pupae. Within one day, the survival probability of this group dropped to 0.90. By day 11, the survival probability of the *masc*-526 injected group continued to decrease to 0.75, also due to several pupae dying without eclosing (Figure 10). By the end of the data collection period, there were 11 surviving *masc*-526 adult *V. cardui.*

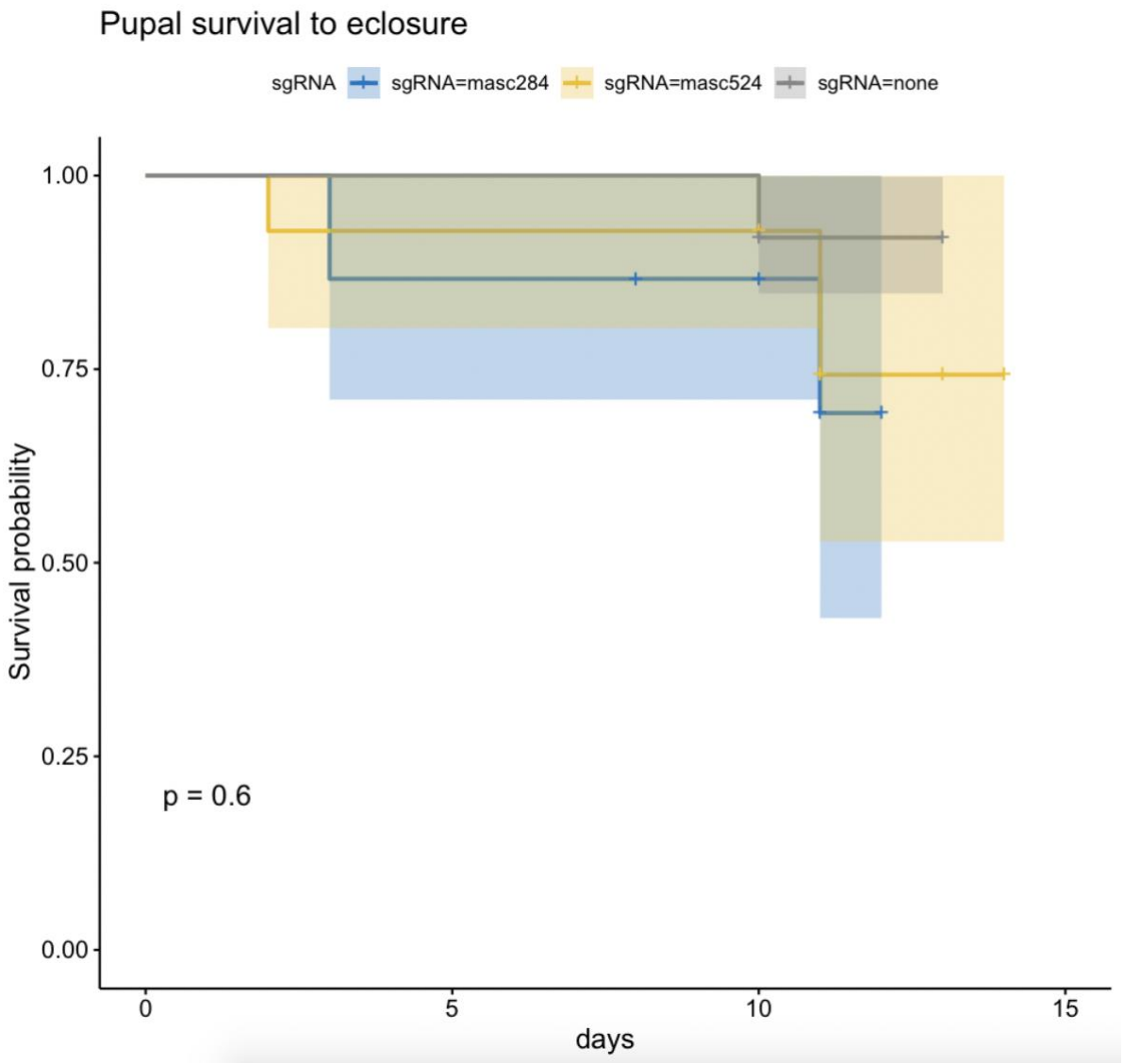

Figure 10. Developmental stage-based survival probability chart from first recorded day of larval stage to pupal stage transition. Larvae were reared in an incubator at 28ºC for injected groups and room temperature for control group.

masc-284 vs. control - Embryo to Adult	X-squared = 14.072, df = 1, p-value = 0.0001759
masc-526 vs. control - Embryo to Adult	X-squared = 15.38, df = 1, p-value = 8.791^{-5}

Table 5. Chi-squared analysis of embryonic to larval stage survival between *masc*-284 injected *V. cardui* and uninjected control group.

3.7: Observation of Foreleg Phenotypes

Individual forelegs were dissected from all 20 *masc* injected animals, and stored individually at room temperature. Analysis of the individual forelegs of each animal was done using a Motic® Moticam 10+ photomicrography camera and Motic Images Plus 3.0® software. Each foreleg was imaged alongside a 4 cm ruler. There was no significant phenotypic variation observed in the masc-284 injected group (Figure 11, Figure 12, Figure 13). All 18 forelegs were comparable to either the wild-type male or wild-type female *Vanessa cardui* phenotype (Figure 3). Phenotypic variation was observed in a small number of *masc*-526 injected individuals (Figure 14, Figure 15, Figure 16), males. The forelegs in Figure 17 were relatively consistent with the wild-type male *Vanessa cardui* phenotype (Figure 3B). Individuals 24, 30, 31, and 34 in Figures 14, 15, and 16 had significantly less hair on their forelegs compared to wild type males. (Figure 3A, Figure 3B). These injected individuals were also noted to be slower and less coordinated in comparison to previous wild-type generations. Overall, the injected individuals did not display any significantly abnormal behavior phenotypic differences when compared to wild-type individuals.

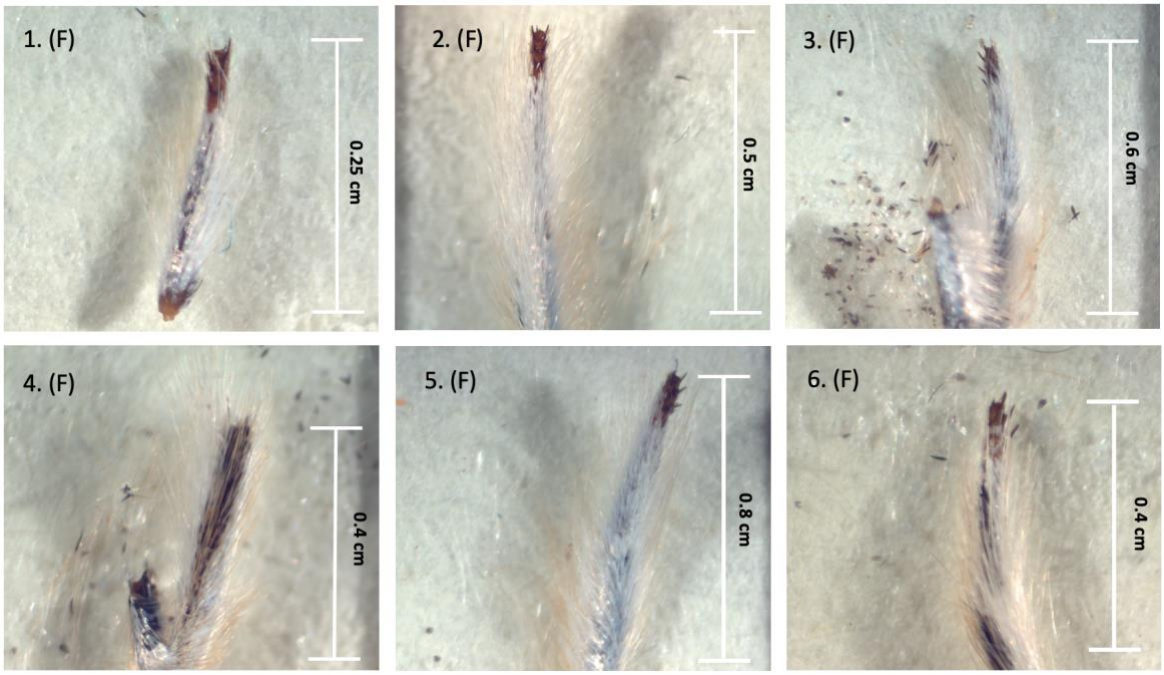

Figure 11. Table of *masc*-284 injected forelegs 1-6. Pictures taken at 2.5X magnification with dissecting microscope.

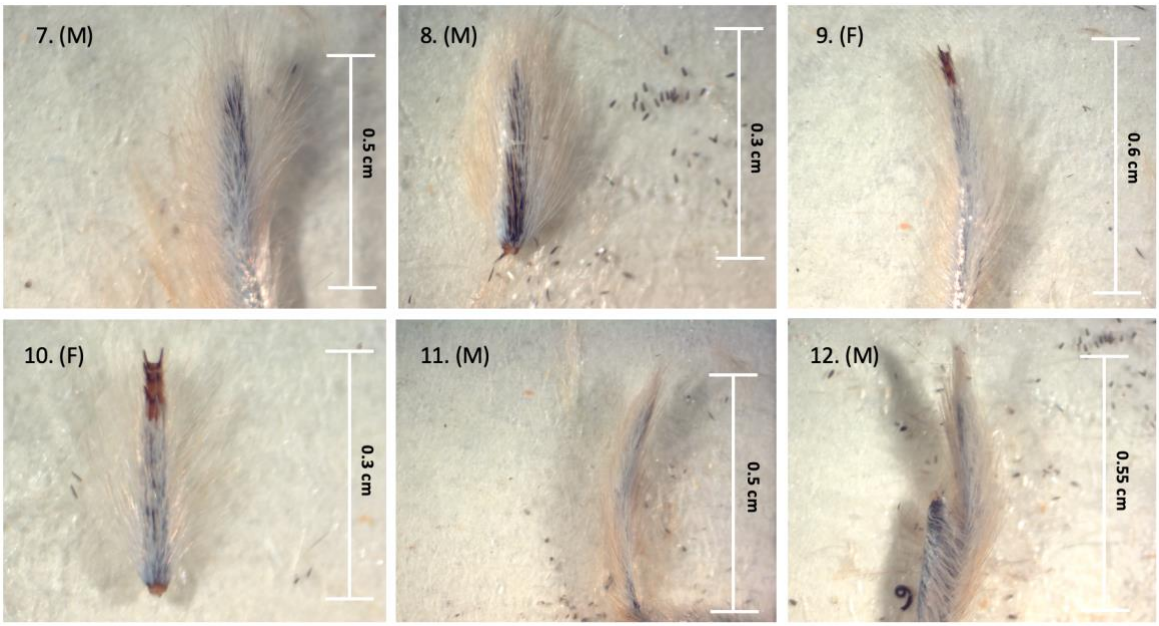

Figure 12. Table of *masc*-284 injected forelegs 7-12. Pictures taken at 2.5X magnification with dissecting microscope.

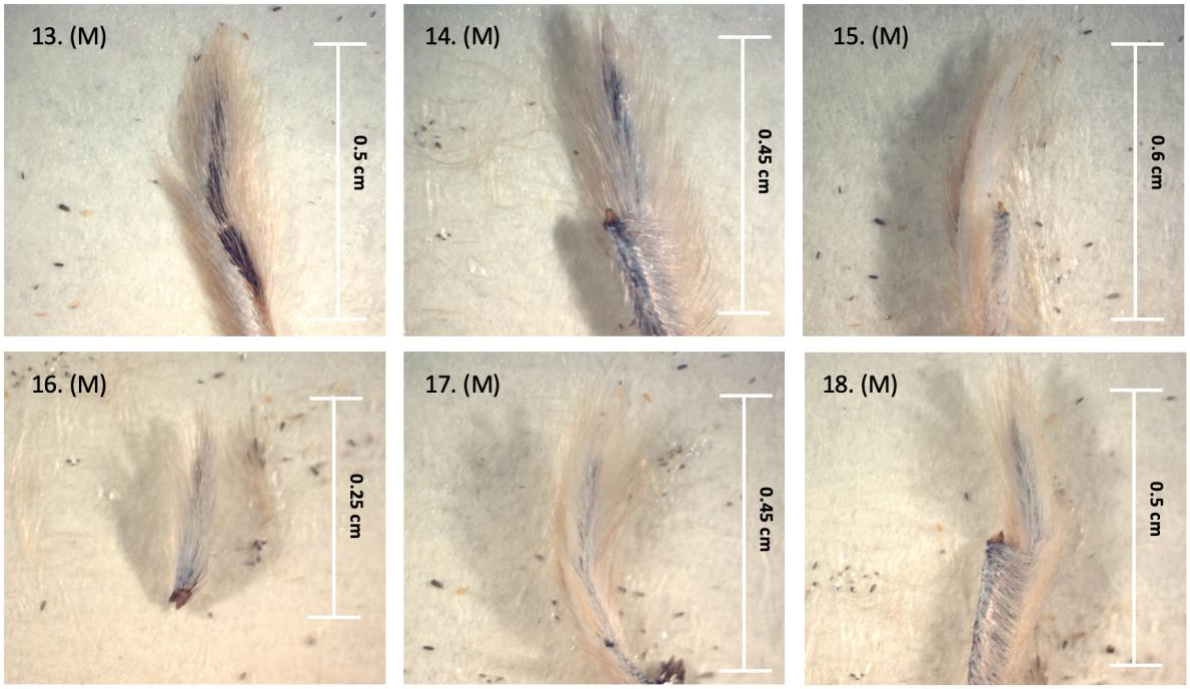

Figure 13. Table of *masc*-284 injected forelegs 13-18. Pictures taken at 2.5X magnification with dissecting microscope.

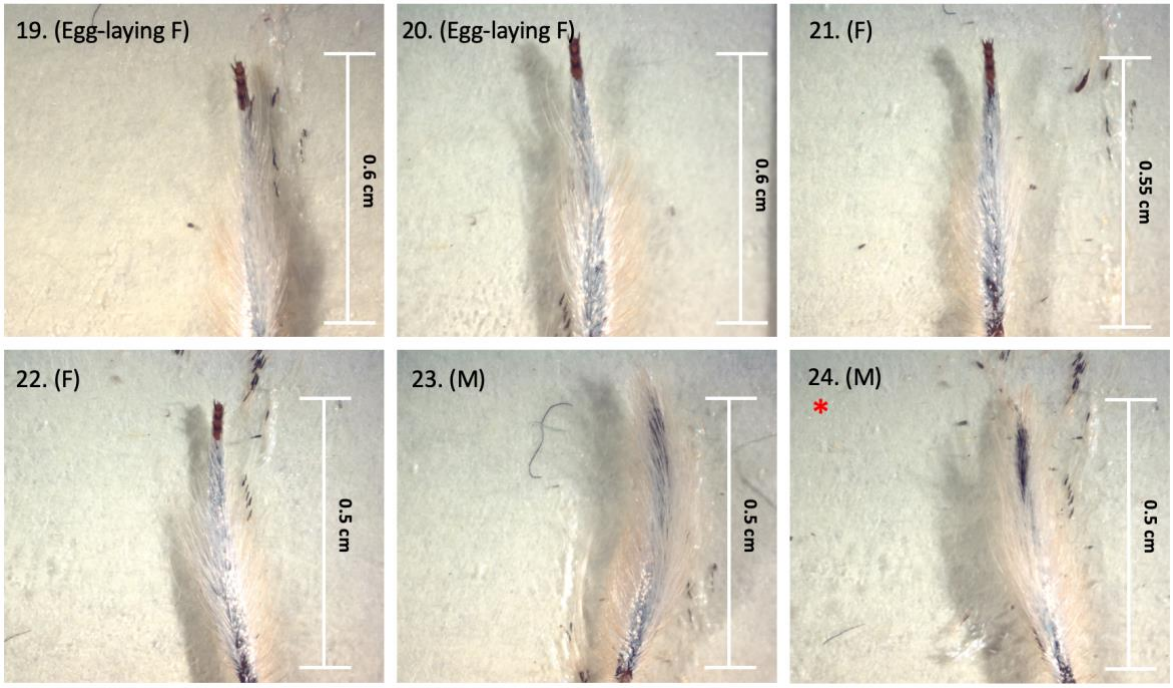

Figure 14. Table of *masc*-526 injected forelegs 19-24. Pictures taken at 2.5X magnification with dissecting microscope. Individual 24 (M) marked with an asterisk to indicate potentially mosaic phenotype.

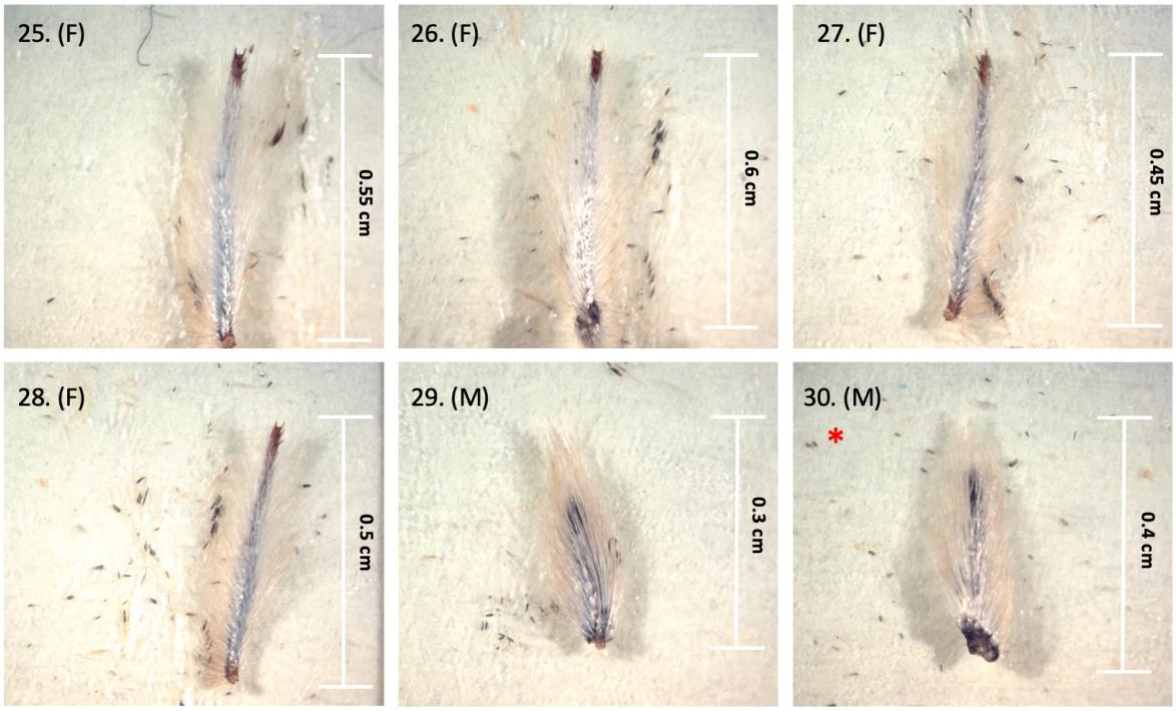

Figure 15. Table of *masc*-526 injected forelegs 25-30. Pictures taken at 2.5X magnification with dissecting microscope. Individual 30 (M) marked with an asterisk to indicate potentially mosaic phenotype.

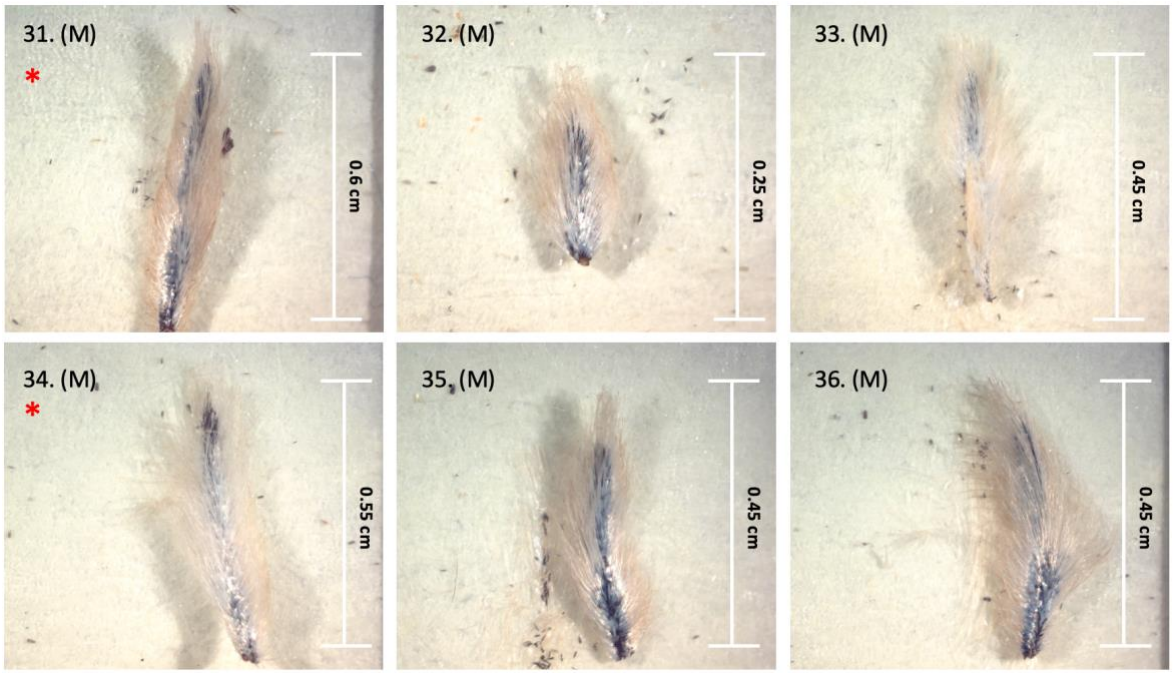

Figure 16. Table of *masc*-526 injected forelegs 31-36. Pictures taken at 2.5X magnification with dissecting microscope. Individuals 31 (M) and 34 (M) marked with an asterisk to indicate potentially mosaic phenotype.

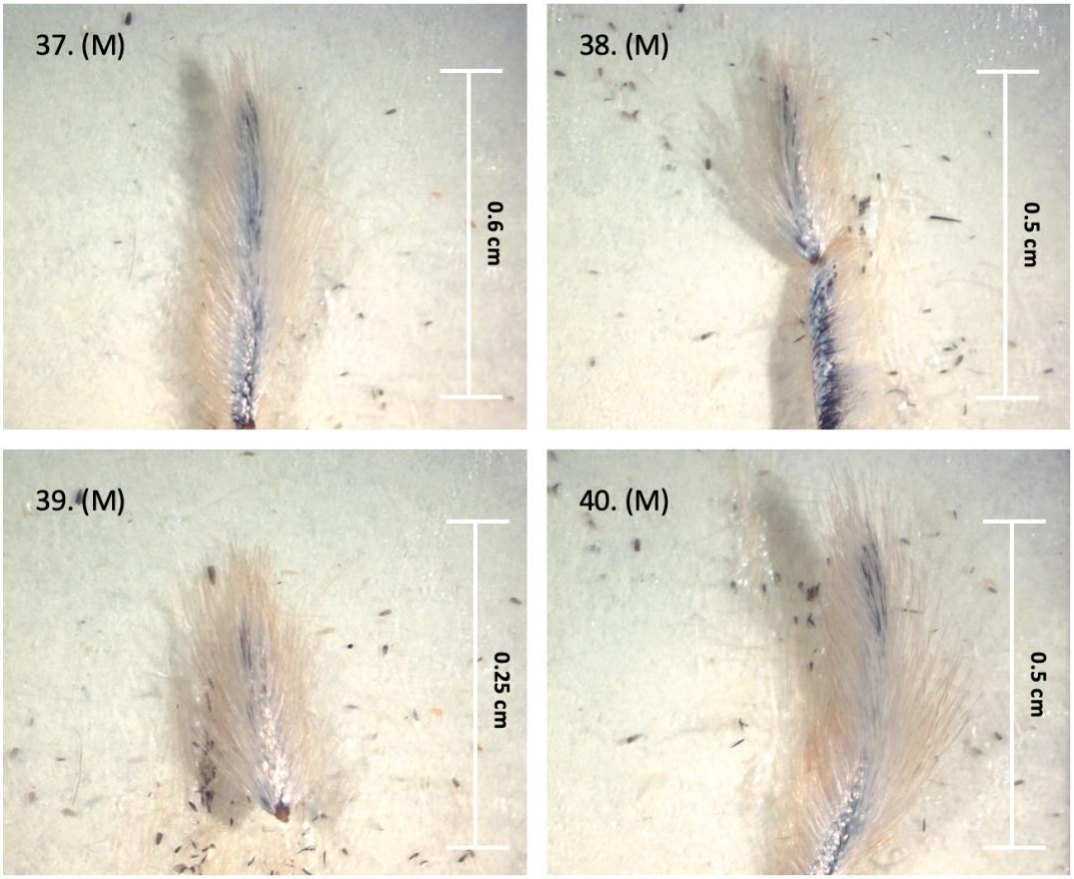

Figure 17. Table of *masc*-526 injected forelegs 37-40. Pictures taken at 2.5X magnification with dissecting microscope.

3.8: 72-Hour Assay: Mating Behavior

Mating assays were performed in a 28ºC incubator following a 48-hour isolation period of all injected individuals selected to participate. Four *masc*-284 injected adults and four *masc*-526 injected adults were selected, and placed in insect terrariums along with a wild-type *V. cardui* adult. There were no instances of mating behavior performed by any of the injected adults in the terrariums during the 72-hour period in which the mating assays were performed (Table 6). Once the mating assays ended, injected adults were placed in netted enclosures. The *masc*-284 group and *masc*-526 group remained separated. The butterflies were left to socialize and observed daily. 6 days after the transition of the butterflies to the netted enclosures, two *masc*-

526 individuals were able to mate and eventually produce eggs (Table 6). Only the date of the observed mating behavior was recorded. A small number of offspring from the injected individuals were collected in a plastic cup and stored in the laboratory at room temperature.

Location	Terrariums				Netted Enclosure						
Day	1	2	3		1	2	3	4	5	6	7
masc-284 x wt	N	N	N	Mating assay ended - *masc*-284 adults placed in netted enclosure *masc*-284 x *masc*-284	N	N	N	-	-	-	-
masc-284 x wt	N	N	N		N	N	N	N	-	-	-
masc-284 x wt	N	N	N		N	-	-	-	-	-	-
masc-284 x wt	N	N	N		N	N	N	-	-	-	-
masc-526 x wt	N	N	N	Mating assay ended - *masc*-526 adults placed in netted enclosure *masc*-526 x *masc*-526	N	N	N	-	-	-	-
masc-526 x wt	N	N	N		N	N	N	N	N	Y	Y
masc-526 x wt	N	N	N		N	N	N	N	N	Y	Y
masc-526 x wt	N	N	N		N	N	-	-	-	-	-

Table 6. Results of 72-hour mating assays using masc sgRNA injected *V. cardui* and wild-type *V. cardui.* Assays were checked three times each day during the 72-hour period: Once at 11:00 am, once at 2:00 pm, and once at 4:00 pm. Due to the absence of any mating interactions observed across the entire assay, the three times were not included in the table. Mating behavior in the injected individuals only was recorded each day with (Y) to indicate the presence of mating behavior or (N) to indicate the absence of mating behavior.

CHAPTER FOUR

DISCUSSION

This experiment aimed to observe the effects of CRISPR knockouts of the *masculinizer* gene on *Vanessa cardui* survival, and to observe whether the function of *masculinizer* is conserved across *V. cardui* as well as other species of Lepidoptera. Because of CRISPR's simplicity and replicability, it can be used on a large group of individuals. The generation of *masc*-knockout *V. cardui* in this experiment had the potential to display mosaic traits, as the microinjection solution components could continuously target and cleave genes throughout the process of embryonic development. Typically, mosaic individuals can show phenotypic differences indicative of CRISPR as well as typical wild-type traits (Mehravar, et al. 2019-2). For *masc*, it was also considered whether *masc* knockouts would prove to be lethal or semi-lethal to embryonic survival. The existence of suspected mosaic individuals suggests that animals with a non-functional *masc* gene still have the potential to survive. However, the respective survival probabilities of the injected and control groups indicates that there may be a significant overarching impact of *masc* knockouts on embryonic survival.

The *masc* sgRNA primers were successfully constructed via PCR, RNA synthesis and RNA purification. The purity of the RNA for the *masc*-284 primer was 1.36 ng/µl. Pure RNA typically has an A260/A280 value of above 2.0 (Thermo Fisher Scientific™). This result for the *masc*-284 primer indicates that there may have been impurities present in the *masc*-284 sgRNA prior to microinjection, which has the potential to interfere with the effects of subsequent experiments, such as the *in vitro* digestion assay and microinjection. The purity of the RNA for the *masc*-526 primer was 2.08 ng/µl, which is optimal for pure RNA, and indicates an acceptable

guide RNA component for microinjection. The sgRNA construction procedure also included two sgRNA primers for *doublesex*, *feminizer*, and *PSI*. However, these primers weren't used in the microinjection procedure. These six primers show varying levels of purity, ranging from 1.22 ng/µl for *fem*-238 to 2.05 ng/µl for *dsx*-196. Based on these observations, it's possible that impurities were introduced at some point during the RNA synthesis or extraction process. It is also possible that impurities present in the *masc*-284 sgRNA primer could have impacted the survival probability data in the *masc*-284 injected *V. cardui* group.

The results of the *in vitro* digestion show the successful cleavage of the *masc* 258 bp PCR product by Cas9-NLS and the *masc*-526 sgRNA primer. Figure 5A displays two potential PCR products that were considered for the digest. The longer PCR product included target sites for both *masc*-284 and *masc*-526. However, it spanned exons, and was not suitable for a PCR reaction that was templated from gDNA. The shorter PCR product was more suitable for the PCR reaction, but it only contained a target site for *masc*-526. Because of this, the *masc*-284 sgRNA, when combined with Cas9, would cut a region that is outside of the 258 bp PCR product. When the PCR was treated with the *masc*-284 sgRNA and Cas9-NLS, splicing did not occur. Therefore, it was impossible to complete the digestion assay of the selected PCR product with the *masc*-284 sgRNA primer. The *masc* PCR product treated with the *masc*-526 sgRNA primer and Cas9-NLS had the capability to be spliced into two distinct fragments with lengths of 190 bp and 60 bp. This evidence of splicing indicates that the *masc*-526 sgRNA is an acceptable candidate for the microinjection process.

Commencing on the day of microinjection, each *V. cardui* egg was observed daily, and all animal deaths were recorded as both *masc* sgRNA injected groups progressed through the four developmental stages (embryo, larvae, pupae, adult). The completed data for the *masc*

sgRNA injected groups was compared with the data of a previous uninjected wild-type generation of *V. cardui*. This generation, consisting of 306 eggs at the start of data collection, served as a negative control.

To visualize the collected data, Kaplan-Meier curves were constructed depicting the data trends during the transition between each developmental stage. is evident that there is a consistent significant difference in animal survival between the two injected groups and the control group across all four major developmental stages. Across all three groups of *V. cardui*, massive loss of embryos occurs. This is relatively typical in wild-type *Vanessa cardui*. Even though the majority of embryos perish before hatching, the minority that survive are still relatively large in number. Despite the overall loss of embryos within all three observed groups in this experiment, the differences in post-embryonic survival between the injected and uninjected groups is apparent in Figure 7. It is possible that the differences in survival could have resulted from the microinjection process, as it is quite invasive to the butterfly embryo and structural integrity of the egg. However, it's also plausible, based on observed phenotypic differences in *masc*-526 sgRNA injected individuals, that the differences in survival can be partially attributed to successful *masc* knockouts and mosaic individuals.

Despite overall loss of embryos across all three groups, the strongest discrepancies in survival probability between the injected and uninjected *V. cardui* groups occurred during the transition from the embryonic stage to the larval stage (Figure 8). Statistical analysis comparing the control group and injected groups indicated a statistically significant difference in the respective survival probabilities of the injected groups compared to the control group. The *masc*-284 injected group, despite not displaying any phenotypic differences in adults, displayed a significant difference in survival probability from the control uninjected group ($X^2 = 17.765$, df =

1, $p = 2.499^{-5}$). The *masc*-526 injected group displayed a similarly significant difference in survival probability when compared to the control uninjected group ($X^2 = 20.176$, df = 1, $p = 7.064^{-6}$). It is worth noting that the control group began with 306 embryos, while each injected group only had 275. This discrepancy in group size may have contributed to the overall difference in survival probability seen at this stage. However, the three groups are considered close enough in size to be compared fairly with the Kaplan-Meier curve and appropriate statistical analysis.

During the transition from the larval stage to the pupal stage, the respective survival probabilities between the control group and injected groups remained significantly different. The *masc*-284 injected group contained 18 larvae at the start of data collection. The *masc*-526 injected group contained 17 larvae. Overall, both injected groups had similar levels of surviving larvae, and weren't affected by extreme differences in sample size. The uninjected group began with 66 larvae. Statistical analysis was performed observing the changes in survival probability from the embryonic stage to the pupal stage. The *masc*-284 group experienced a drop in survival from 18 larvae to 13 pupae. This was significantly different from the uninjected group, in which 66 larvae became 50 surviving pupae ($X^2 = 12.939$, df = 1, p-value = 0.0003218). In the *masc*-526 injected group, only 13 of the surviving 18 larvae successfully pupated. This was also significantly different from that of the control group ($X^2 = 14.096$, df = 1, p-value = 0.0001738). There was one outlier in this particular data set. One *masc*-526 injected larva did not pupate for an unusually long time, but remained alive. It was observed daily to see whether it would eventually pupate, but it perished without a pupation attempt after 10 days of observation. During the transition from the pupal stage to the adult stage, the loss of individual animals in the injected groups became less prevalent. In the uninjected group, 50 pupae were placed into the

styrofoam pupation chamber, and 46 wild-type *V. cardui* adults eclosed. In the *masc*-284 group, 13 pupae went into the pupation chamber, and only 9 adult butterflies eclosed. Statistical analysis found a significant difference between the survival probabilities of the *masc*-284 injected group and control group ($X^2 = 14.072$, df = 1, p-value = 0.0001759). The *masc*-526 injected group began with 13 pupae, and ended with 11 surviving adults. This group's survival probability was also significantly different from the control group ($X^2 = 15.38$, df = 1, p-value = 8.791^{-5}). If an animal perished before the end of data collection, the event was recorded as a tick mark on each Kaplan-Meier curve. These tick marks are most visible in Figures 9 and 10, in which individual deaths were easier to record among the smaller number of *V cardui* in each group.

All individual forelegs from injected *V. cardui* were dissected after the animals perished naturally. Forelegs 1-18 came from the 9 butterflies in the *masc*-284 injected group. Each group of two forelegs was removed from one butterfly. Forelegs 1 and 2 originated from the same injected butterfly. Forelegs 3 and 4 originated from a different injected butterfly, and so on. Forelegs 24, 30, 31, and 34 displayed a significant phenotypic difference. Wild-type male *V. cardui* forelegs are typically very hairy, and the foreleg tip is concealed entirely. Forelegs 24, 30, 31, and 34 had significantly less hair, providing visibility of the foreleg tip. None of these forelegs had any indication of female-specific tarsal claws. Based on the observations, it is apparent that *masc*-526 injected male butterflies were the only group that contained suspected mosaic individuals. This is consistent with the observation that *masc*-526 was the only sgRNA primer that was predicted in the *in vitro* digestion to produce successful CRISPR activity. Genotyping has yet to be completed, and will determine if the phenotypic differences and survival differences are attributed solely to the microinjection process. There is also the presence of an egg-laying female possessing forelegs 19 and 20 in the *masc*-526 injected group. The egg

laying was preceded by an isolated instance of mating between a *masc*-526 injected mosaic male and female 13 days after all *masc*-526 adults had eclosed. No mating behavior was observed during the mating assay, but once the butterflies were placed together in a netted enclosure, they began to socialize. The success of *Vanessa cardui* courtship, and the presence of healthy eggs laid by the *masc*-526 female demonstrate that CRISPR activity on *masc* does not seem to affect mating behaviors or reproductive activity in *Vanessa cardui* mosaic individuals. It might be possible that the *masc*-526 egg-laying female and the male involved in courtship did not receive successful CRISPR-Cas9 activity, and that they are wild-type individuals. However, it is important to note that once properly socialized, injected butterflies were able to mate normally.

While collecting the data for this experiment, it was impossible to construct a positive control group of 275 Cas9 blank injected butterflies with no additional sgRNA primer. The generation that was used to produce the eggs for microinjection only laid around 500 usable eggs, and it was impossible to collect enough to produce a positive control alongside the two experimental groups within the time limit allotted for this experiment. In future studies, a positive control would provide more insight on whether the differences in animal survival probability were primarily due to the successful knockout of the *masculinizer* gene, or solely the invasiveness of the microinjection process.

In constructing the Kaplan-Meier curves, there were some differences between the data collected in this experiment, and data typically represented in a Kaplan-Meier curve. The *V. cardui masc* survival data had been split into three sections to delineate the transitions between the four major Lepidopteran developmental stages. Typically, Kaplan-Meier curves are used in medicinal research to measure survival following drug/clinical trials, particularly for novel cancer treatments (Goel, et al. 2010). The three Kaplan-Meier curves provided the best

representation of the specific quantitative data collected for this experiment, but the additional consideration of developmental stages in the data for this experiment provided an obstacle to consider constructing the Kaplan-Meier curves.

In observing phenotypic differences in injected individuals, it is probable that some of them will display genotypic differences that can be observed with the proper procedure. Evidence of successful CRISPR activity in mosaic butterflies could be confirmed with Sanger Sequencing of genomic DNA extracted from the forelegs or genitalia of injected adults.

After the mating assay, injected adults were allowed to roam around a netted enclosure, as opposed to the insect terrariums. Forelegs were observed after all the injected adults had naturally perished. Because of this, it is possible that some phenotypic differences in the forelegs could be attributed to the age and activity of the butterflies.

This research was done to explore the conservation of the *masculinizer* gene across Lepidopterans. *Vanessa cardui* injected with the *masc* sgRNA primers displayed phenotypic differences in male forelegs, including the absence of hair, which is associated with female *V. cardui*. These mosaic individuals suggest that *masculinizer* acts as a regulator for male *V. cardui* development, and the absence of *masc* could contribute to decreased embryonic survival, as well as decreased adult fitness in mosaic male individuals. The embryonic survival differences witnessed and recorded in this experiment could be the result of *masculinizer* as an initiator for dosage compensation in males, and its absence resulting in hyper-upregulation of Z-linked genes in Lepidopterans. Future studies involving Lepidopteran sex determination should observe potential effects of other conserved genes, such as doublesex or feminizer, on embryonic survival and adult fitness.

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APPENDIX

Github: *masc* survival analysis raw data and RStudio code
https://github.com/aphanotus/VanessaMasc

HackMD: Various procedures from the Angelini Lab
https://hackmd.io/@EcoEvoDevoLab/AngeliniLab
https://hackmd.io/@aphanotus/bi332f19

B. mori target sequence used for *masc* primer building

AAAACTGAAGTTGCTAGGTACTTGTCTGTCACTTTTTACTCTAATACTTTTGAGTTAAACTATATCAACATGGCT
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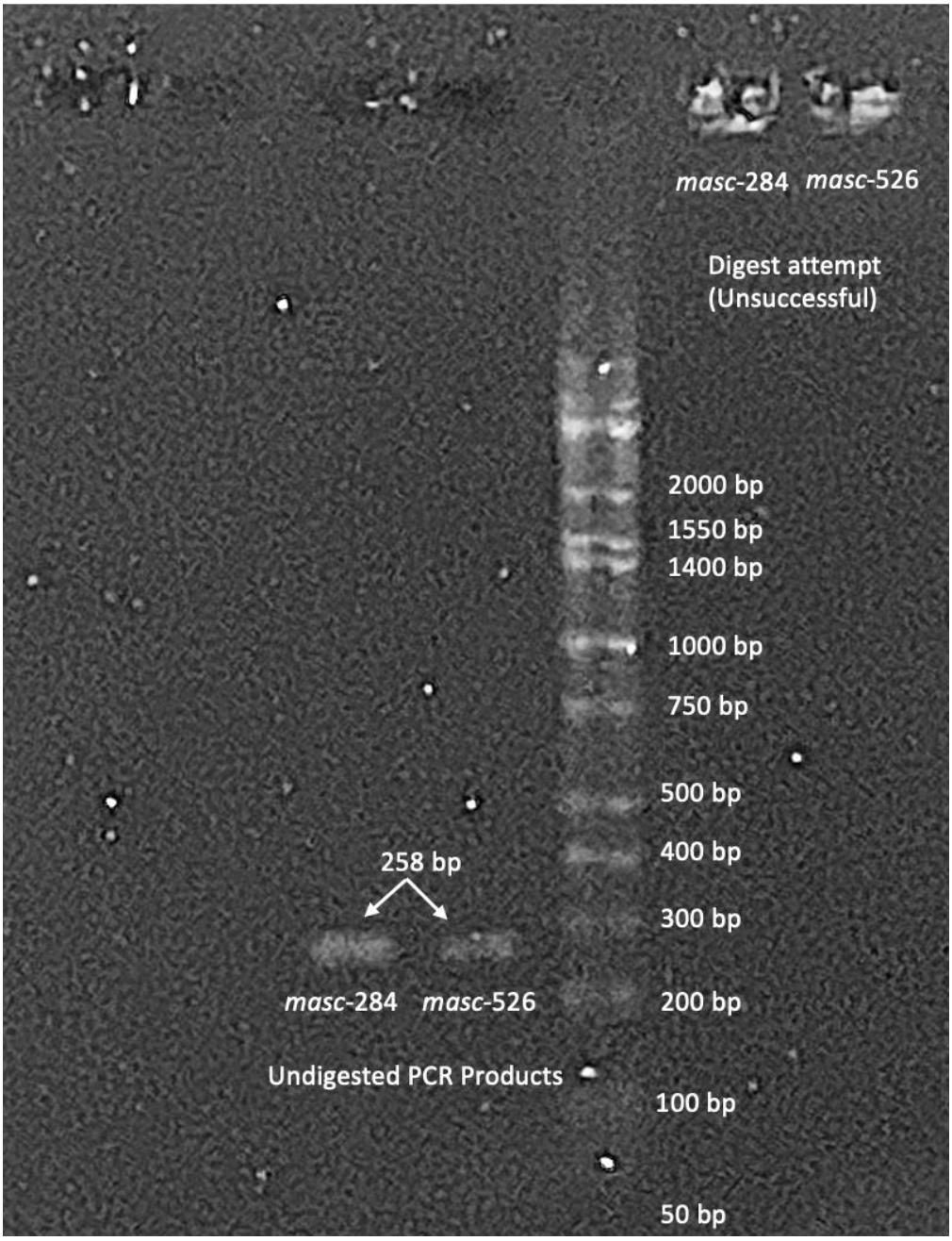
TCGAATGTAATAGTTGTAAGGGGAAAAACTAG

in vitro digestion of DNA with Cas9 - First attempt



Vanessa cardui visible mating behaviors. (A) male butterfly interacting with female butterfly by pointing abdominal posterior in the direction of the female. (B). Successful female and male mating pair.