Testing Efficiency of a Native Lethal Transgenic Line of *Drosophila* as Insect Population Control

Word Count: 4952

Introduction

Insect population control has been an issue in many environments. Methods for such population controls vary widely in their applications and efficacy. Many existing methods such as the sterile insect technique and lethal transgene utilize genetics and mating patterns to manipulate the populations, however there are several drawbacks to those methods. This research is an introduction and test of a new method of population control through a lethal transgene introduced to the native species via marker assisted backcrossing on fruit flies (*Drosophila melanogaster*). The efficacy of the marker assisted backcross will be assessed through a comparison of the fertility of the transgenic line in comparison to the wild type.

Literature Review

A common process for studying genetic variation in plants and insects is one called backcrossing, specifically marker-assisted backcrossing (Frisch, 2005). This is a process of integrating a transgene into an organism's genome through crossing two populations repeatedly. These two populations are the parents; the Recurrent Parent is the organism from the population in which the target transgene is being integrated, the Donor Parent is the laboratory grown line containing the transgene (Hasan et. al, 2015).

Genetic Variation and Backcrossing

The backcrossing process is most efficient and simplified when it is conducted on populations with little genetic variation (Chisholm et. al, 2019). This is because genetic variation introduces unknowns into the genome. This process has a very precise location (target loci of the

transgene) that is being traced and isolated, so the more genetic variability, the more likely the inheritance of this loci is to be disrupted.

The backcrossing process is so vulnerable to genetic variability because of the existing unpredictability due the event of crossing over for each mating line. Crossing over is the process of random exchange of genetic information between the chromosomes of the two parents in the creation of the DNA of the offspring (Mather, 1938). The goal of the marker-assisted backcross is to use this exchange of genetic information to exchange the entirety of the background genome of the Donor Parent with that of the Recurrent Parent without disrupting the target loci containing the transgene (Hasan et. al, 2015). This random exchange gets more complex when there is more genetic variation and therefore fewer progeny inherit the entirety of the target loci. The laboratory grown lines have a much simpler genome in comparison to the native lines. Therefore, the greater genetic variation of the native lines may affect the backcrossing process due to the unpredictability of the genome and the mutations that the offspring may carry (Chisolm et. al, 2019). Therefore the efficacy of backcrossing the lethal transgene in native, complex, *Drosophila* versus the laboratory grown *Drosophila* needs to be analyzed in order to assess the efficacy of the method for applications in insect population control methods.

This backcrossing process has many applications, many of which include insect population controls. There are two main insect population controls that utilize backcrossing: the sterile insect technique (Hendrichs, 2009) and the lethal transgene method (Alphey et. al, 2002).

Sterile Insect Technique

The sterile insect technique is a common population control method that requires alteration to the organisms' genomes as well as disrupting the biological processes without

disrupting the mating processes. Dr. Pérez-Staples from the Instituto de Biotecnología y Ecología Aplicada uses this sterile insect technique to track sterile, transgenic organisms and their mating efficacy. This involves the release of mass sterile males to mate with wild type females to track the pre and post copulatory success of the females' mating failure (Pérez-Staples et. al, 2012). This study used native populations as the sterile males that were tested as population controls which allowed for optimal mating efficacy when it comes to variation in genome. However, the gene that was used to sterilize the males often causes unwanted side effects such as blindness, hyper-sensitivity to temperatures, and other defects that may lower their mating competitiveness (Thomas, 2000). This method was successful in the use of backcrossing to introduce the sterile gene, however the sterile gene itself was flawed due to the negative effects inflicted on males.

Lethal Transgene Method

Another mode of insect population control utilized a conditionally lethal transgene to inhibit the growth of the native populations. This lethal transgene is otherwise known as the Head Involution Defective, or hid gene (Brody, 2011). The hid gene controls the stage in embryo development known as head involution. This process allows the initiation of apoptosis (programmed cell death) in the specific line of cells that attach the head to the chest of the *Drosophila* during the early stages of development. The defect in this gene inhibits apoptosis, thus preventing the head from detaching from the chest, halting further development of the embryo, and effectively killing the *Drosophila* (Yoo et. al, 2016). A study conducted by Professor Luke Alphey and his team from the Pirbight Institute used this gene as population control for insects by injecting the lethal transgene into a laboratory line of *Drosophila* and releasing them to mate with the native population (Alphey et. al, 2002). This allows the females

to mate with the transgenic males rather than the native males, thus decreasing the amount of viable offspring the females produce.

There is an issue with the lethal transgenic method due to the limitations of the specific line of *Drosophila* in which the transgene is inserted. The line that is used in this method is created in the laboratory and is therefore much less complex than the native *Drosophila* (Thomas, 2000). This means they are not as competitive as the nativel line for mating and therefore are not as effective for population control. One such laboratory line of *Drosophila* is called the DH1 line. The H1 protein, or Histone 1, is a simple protein aiding in many biological processes that has many variants. The DH1 population only contains one variant and therefore provides little genetic variation (Vujatovic et. al, 2012). This provides a very stable organism to test in a lab setting, however, when applied to the native environment, the systems of the DH1 lines may be too simple to compete with the complex native lines.

Backcrossing

In order to eliminate such limitations, the transgene can be integrated into the genome of the native species. Backcrossing is a widely accepted approach used to introduce a transgene into the native population (Hospital, 2005). For the purposes of this study the Recurrent Parent is the line native to Lexington, Kentucky (P814) and the Donor Parent is the laboratory grown line (DH1) with the inserted lethal transgene.

As Dr. Hasan, from the University of Putra Malaysia describes, the backcrossing process starts with a cross between the Recurrent Parent and the Donor Parent. The progeny of this cross are then mated with the Recurrent Parent. This trend of mating the progeny of the previous generation with the Recurrent Parent is continued until the backcross 6 filial 1 generation

(BC6F1). At this point the crosses are filtered to identify one confirmed double homozygous line, a process that may vary between scientists, and is further discussed in the methodology. A double homozygous line is one that contains both alleles coding for the driver and effector genes (phenotypic markers for the presence of the loci). The goal of this process is to obtain a line that is completely double homozygous for the transgenes and contains as close to 100% of the Recurrent Parent's background genome as possible (Hasan et. al, 2015). This backcross process is used in this study to integrate the lethal transgene into the native population as a new potential method of insect population control.

New Insect Population Control Method

The gap in this research lies in the connection between the sterile insect technique and the lethal transgene method. The advantages of the sterile insect technique lie in the introduction of the sterilizing gene into the native line rather than the laboratory grown line. However, the sterile technique is not as effective as the lethal gene may be because the gene that sterilizes the male may have other repercussions that affect their mating patterns (Thomas, 2000). The lethal transgene method uses a gene that is effective in eliminating the offspring of the transgenic parents yet doesn't affect the full-grown adults. However, the disadvantage to this method lies in the fact that in the existing studies for this method the lethal transgene has only been applied to laboratory grown lines (Thomas, 2000). These lines have a much simpler genome from those in the native populations and therefore are not as competitive when mating with the native males.

The disadvantages of these methods can be eliminated to combine the advantages in a new method. This method will follow a similar process to the sterile insect technique but utilizing the lethal transgene. This will establish a possible population control method that avoids

the limitations of both of these established techniques. The line of *Drosophila* that are transgenic will be tested for mating efficacy against four populations: P814, DH1, Oregon R, and BC6F1 Oregon R. The P814 population is a wild type line originating in Lexington, Kentucky. The DH1 population is a wild type line originating in the laboratory with little genetic variation. The Oregon R population is a wild type line that also originated in the laboratory with higher genetic variation. The BC6F1 Oregon R population is the controlled transgenic line. This line has gone through the same backcross as the experimental BC6F2 P814 line, however the Recurrent Parent is the laboratory, Oregon R line, rather than the native, P814, line. Through analyzing the fecundity and fertility of the native transgenic line versus the wild type lines, the plausibility of the native lethal transgenic *Drosophila* method of insect population control can be assessed.

Methodology

I was able to work in an entomology lab at a local university. Through this lab I was able to utilize the college resources and lab space to conduct my research under the supervision of my expert advisor, a PhD candidate at that University.

Backcrossing

The research will be conducted through the use of backcrossing, a process that was explained in the literature review. This backcross will initially cross the Recurrent Parent, originating in Lexington, KY, with a Donor Parent, originating in the laboratory to produce the F1 (filial 1) generation. The backcross for this study is specifically a marker assisted backcross,

meaning the target loci containing the lethal transgene will be marked with two visible (phenotypic) transgenes. Each marker produces a specific phenotype, as shown in **Figure 1**. The first marker is a driver that causes the body of the *Drosophila* to shine red as seen in the top middle image. The second marker is the effector that produces a cyan fluorescent phenotype in the *Drosophilas'* eyes as seen in the top right image. The images on the bottom are a comparison to a wild type that does not carry the lethal transgene or these two markers.

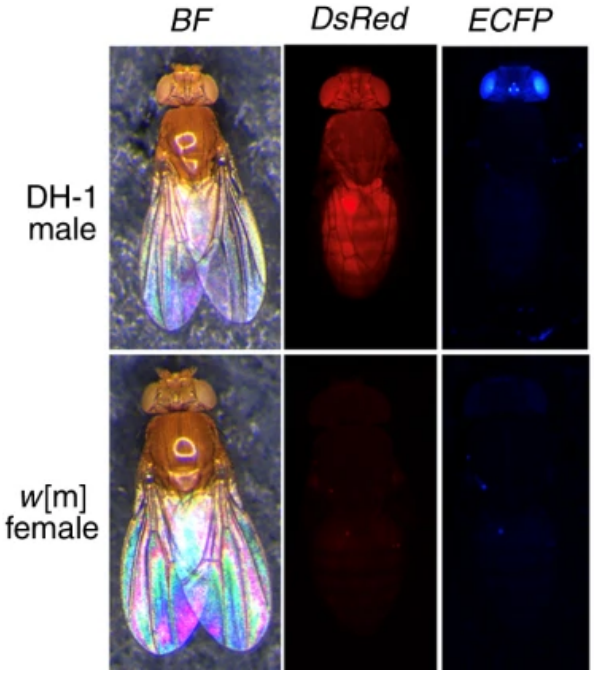

Figure 1 The driver and effector phenotypes of a DH1 male (top) and *w*ild type female (bottom) (Zhao et. al, 2020).

Through these markers the presence of the entire target loci can be tracked. This is necessary because the process of crossing over is unpredictable and it is possible that during the separation of the nucleotides the loci could be broken apart (Mather, 1938). The goal of this process is to introduce the entire background genome from the Recurrent Parent into the Donor

Parent line with an end line containing the entire target loci surrounded by the genome from the Recurrent Parent. This means the end line will be as close to 100% double homozygous (presence of both driver and effector) as possible and the background genome is as close to 100% of Recurrent Parents' genome as possible.

After each cross, DNA extraction and a PCR (Polymerase Chain Reaction) will be performed to identify the lines that possess the target gene. DNA extraction is a process of purifying the tissue by breaking down all macromolecules and leaving nucleotides to isolate the DNA. A proteinase solution, various buffers, and ethanol are used to purify and extract the DNA. The PCR is performed on the extracted DNA to isolate the target gene sequence by using primers specific to the sequence. An electrophoresis gel is then used to identify the lines that contain the target gene sequence. According to the National Library of Medicine this is the best process for identifying the DNA sequences in organisms and are basic procedures used in most molecular laboratories (Gupta, 2019). DNA extraction and PCR are used in this study to identify the lines that are to be continued with the backcross by eliminating those that do not possess both the driver and effecter marker transgenes.

The backcrosses are conducted through a series of crossing fluorescent female offspring from the previous generation with a Recurrent Parent. There was a period of time during these crosses in which the lines were left to inbreed out of an extended period of time in one vial. This occurred during backcross 4, thus producing BC4F2 (Backcross 4, filial 2) rather than the expected BC4F1. This did not change the outcome of the crosses, yet should be noted if this process were to be replicated.

The crosses between the fluorescent female offspring from the previous generation and the male Recurrent Parents are continued until the 6th backcross which would produce the BC6F2 (Backcross 6 Filial 2) line. Once this line is achieved, the BC6F2 line will be put through a series of filters to find one line that is confirmed to be double homozygous for the transgene. The first filter occurred when the phenotype of the last generation was analyzed to identify the *Drosophila* that are fluorescent. The second filter occurred when 100 of the virgin, fluorescent *Drosophila* from this last backcross, BC6F2, were collected and placed into non-tetracycline vials. The tetracycline that is in the food in the vials inhibits the expression of the lethal gene, therefore allowing the offspring to develop past the embryonic stage. In the absence of tetracycline, the progeny carrying the lethal transgene will not develop past the embryonic stage.

In the non-tetracycline vials the 100 virgin females were each crossed with male from a different previous cross. The parentals from these new crosses will be transferred to tetracycline vials once they have successfully mated in the nn-tetracycline vials. This will allow the line to continue with viable offspring for further analysis. Once the offspring from these crosses have emerged, the presence of non-fluorescent survivors will indicate that both of the parentals were heterozygous recessive and that line will be removed. Those that remained are considered candidates for the final double homozygous line. The parentals of these candidates were either 50% homozygous for the transgene and 50% heterozygous or 100% homozygous. This is because if all of the offspring carried the lethal transgene and did not make it past the embryonic stage, Mendelian genetics states that at least one parent would have to be heterozygous (Collins et. al, 1989). If the offspring were observed to have survived the embryonic stage and emerged as adults yet presented the fluorescent phenotype, then these lines were continued as candidates. Those instances could have been due to human error as the offspring could have had some excess

tetracycline left in their systems if the parentals were not drained of the tetracycline for a sufficient amount of time. Therefore the offspring could carry the lethal transgene yet it wouldn't have been expressed due to the presence of the tetracycline. Those cases were treated to have the same possible genotypic parentals as those that observed no emergence.

A summary of the backcross process is displayed in **Figure 2** from Dr. Hasan. The figure shows the increased proportion of the genome that is recovered from the Recurrent Parent for each backcross. The goal for this cross is to reach a line where this percentage is as close to 100% as possible and is double homozygous for the lethal transgene. This means that the genome of the final line would be almost entirely recovered from the Recurrent Parent and homozygous for both the driver and effector marker genes.

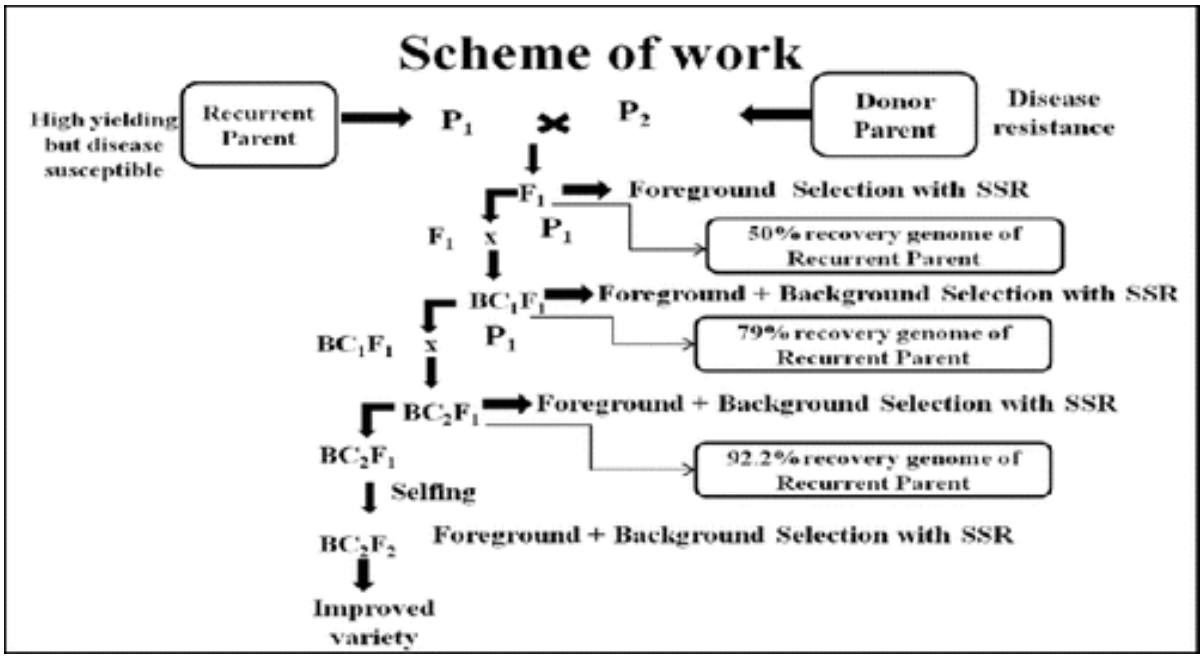

Figure 2 *Scheme of Work* (Hasan et. al, 2015)

This backcrossing process takes several months to complete as each cross takes 12-14 days to complete and analyze. After the final cross there are several candidates for lines that would be truly double homozygous for the transgene, however there were none that were definitively double homozygous. This was because none of the progeny from the second generation females crossed with P814 (filter 3) were observed to be 100% fluorescent. There are ways to complete this process and have one definitive line, however with the timeline for this research, 5 candidates for double homozygosity were selected to further analyze and compare fertility with the wild types.

Comparison of fertility

The final stage of the research process is to test the fertility of the transgenic, native *Drosophila* (BC6F1 P814) versus the wild type lines. This will be conducted through observing 5 crosses of each lineage: 5 from the DH1 population, 5 from P814, 5 from Oregon R, 5 from BC6F1 Oregon R, and 5 from each of the 5 candidates for the BC6F2 P814 population. These crosses are evaluated based on the quantity of emergence of the progeny as well as the time of emergence (fertility per day per female). The lines with the highest fertility are the lines that can be considered as the most competitive for mating within their respective lines. The competition between the lines will not be evaluated, as the lack of a confirmed double homozygous line would alter the results from the true values that would be seen of a confirmed line. Instead, the lines' fertility was evaluated within their own line and compared to the others independently.

This methodology is proven most effective as it ensures the use of a line that is double homozygous for the target transgene and contains the background DNA from the Recurrent

Parent as well as fully analyzes whether the experimental transgenic *Drosophila* are more or less efficient in mating than the wild types.

The predictions for the fecundity of each line are shown in **Table 1**. “No growth” indicates the prediction that there may be embryos but they will not develop past the larval stage and “Growth” indicates the prediction of full development throughout the line. P814 and Oregon R are the only 2 lines that were hypothesized to observe growth in the absence of tetracycline. They are the only two lines that do not contain the lethal transgene, therefore the tetracycline is not necessary for the viability of their offspring. The other 3 lines do carry the lethal transgene and will not have viable offspring without tetracycline. All 5 lines were hypothesized to observe growth in the presence of tetracycline as the tetracycline does not inhibit development of any line, therefore each line should develop normally.

	No-Tetracycline	Tetracycline
BC6F1 (OR)	No growth	Growth
BC6F2 (P814)	No growth	Growth
P814	Growth	Growth
DH1	No growth	Growth
Oregon R	Growth	Growth

Table 1 predictions for development of each line in presence and absence of tetracycline.

Results

Backcrosses

From the 100 lines that were crossed in non-tetracycline vials, 41 were selected as candidates to move on to the next stage of mating with P814. This is shown in **Table 2** under the yellow section for non-tetracycline vilas and “False” column for “Emergence” and “True” column for “Some Fluorescence”.

		True	False	N/A	Candidates
Non-Tetracycline Vials	Emergence	73	29	0	41
	Some Fluorescence	12	59	29	
Crossed with P814	Emergence	22	13	65	2
	100% Fluorescence	2	20	78	
Progeny Crossed with P814	Emergence	21	3	76	0
	100% Fluorescence	0	21	79	

Table 2 Expected values for number of vials that had emergence and fluorescence.

From the 100 lines tested in non-tetracycline vials, 56 were expected candidates for the next cross. Meaning 44 vials were expected to have emergence and 56 were expected to have no emergence based on the probability of having both homozygous parents as seen in **Table 3 and 4**. These probabilities were developed from Mendelian genetics and laws of inheritance (Collins et. al, 1989). From these values the chi square value for a goodness of fit test was calculated to be 0.0025. This value is much smaller than the 1% significance level, therefore there is statistically significant evidence to show a difference between the observed number of vials with emergence and the expected. There are a number of factors that could contribute to this discrepancy such as the possibility that the heterozygous *Drosophila* were more competitive in mating than the homozygous *Drosophila*. Another possibility could be that the homozygous *Drosophila* take longer to develop and therefore there were not as many double homozygous selected as part of these 100 random samples as expected due to the timeline of the sampling.

	Female	Male	Total
A	2/3	1/3	2/9
B	1/3	2/3	2/9
C	1/3	1/3	1/9
D	2/3	2/3	4/9

Table 3 Expected proportion of males and females for each genotype.

	Female	Male
A	Dr/Dr ; Ef/Ef	Dr/+ ; Ef/+
B	Dr/+ ; Ef/+	Dr/Dr ; Ef/Ef
C	Dr/Dr ; Ef/Ef	Dr/Dr ; Ef/Ef
D	Dr/+ ; Ef/+	Dr/+ ; Ef/+

Table 4 genotypes for conditions in **Table 3**. “Dr” denotes presence of the driver allele, “Ef” denotes presence of the effector allele, and “+” denotes wild type allele.

From the 35 vials that were viable to be continued from the 41 candidates, 2 were observed to be 100% fluorescent as shown in **Table 2** under the “100% Fluorescence” row and the “True” column in the blue section of crosses with P814 males. This means that all of the progeny from these 2 vials possessed the driver transgene. However, upon analysis of the cross between the progeny of these lines and the P814 line, the purple section of **Table 2**, there were no lines observed to have 100% fluorescent progeny. This means that, even though there were 2 lines that produced a 100% fluorescent progeny, that result was not confirmed in the second half of the 3rd filter, therefore one more trial for filter 3 would need to be conducted. As a result, 5 candidates for double homozygosity were used in place of one confirmed double homozygous line.

5 crosses of each population (BC6F1 OR, P814, DH1, OR, and 5 candidates of BC6F2 P814: 25, 34, 36, 45, and 49) were assessed for presence of embryos, larvae, and pupi after 1 week and survival of adults after 2 weeks. The results of this analysis are shown in **Figures 3-6.**

The presence of embryos and larvae indicates the fecundity of the population while the presence of emergence or high survival of adults indicates high fertility.

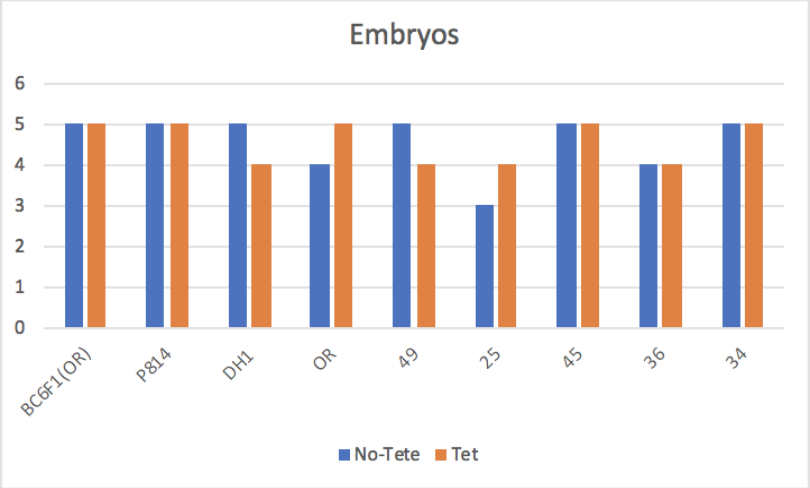

Figure 3 Number of vials with the presence of embryos after one week

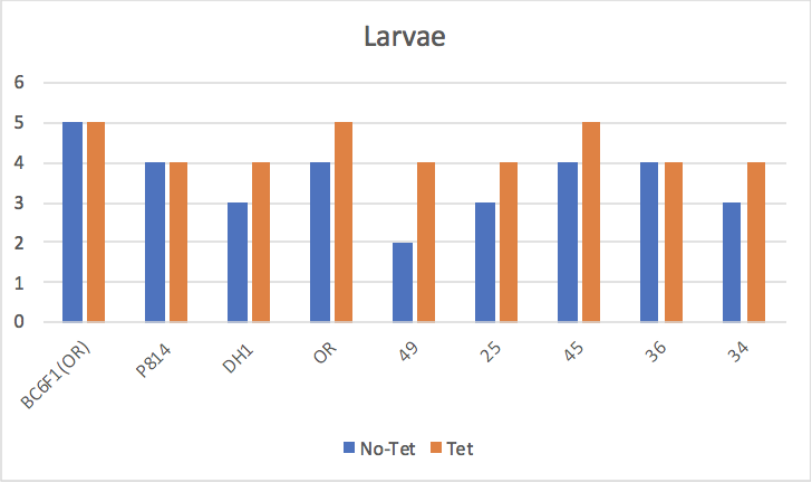

Figure 4 Number of vials with the presence of larvae after one week

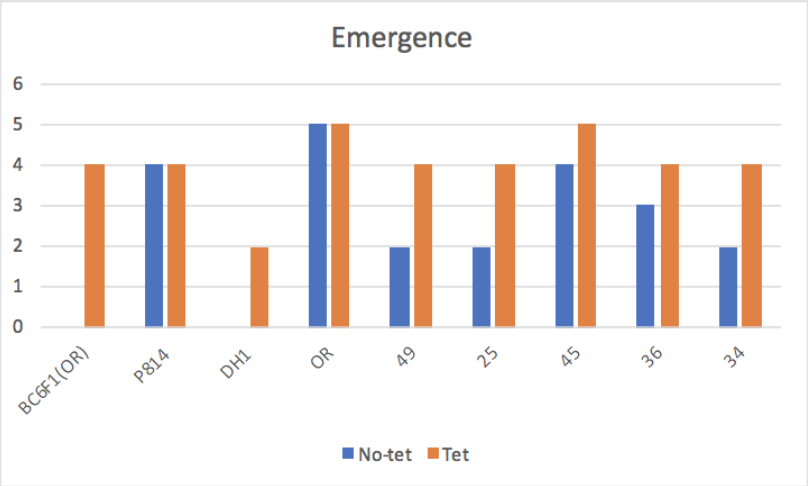

Figure 5 Number of vials with the emergence after two weeks

Each population presented high fecundity as at least 4 lines in each population observed the presence of embryos in a tetracycline rich environment and at least 3 in each population in a non-tetracycline environment. However, the fertility of each population varies from each other and between presence and absence of tetracycline. The number of lines observing emergence is lower for all populations except P814 and Oregon R in the non-tetracycline environments.

The fertility per day per female for each population is shown in **Figure 6**. The fertility per day per female shows the number of viable offspring one female will produce per day. This represents the mating efficiency within each line as well as the timeline for their mating processes. According to **Figure 6** line 36 showed the greatest fertility per day per female in the presence of tetracycline and the Oregon R population had the highest fertility per day per female in the absence of tetracycline. This means that in the presence of tetracycline (when the expression of lethal transgene is inhibited) line 36 from the transgenic, native populations (BC6F2 P814) is the most fertile and in the absence of tetracycline the Oregon R population is

the most fertile. However, in comparison to the wild type, native population (P814), all 5 of the candidates had a higher fertility in both presence and absence of tetracycline.

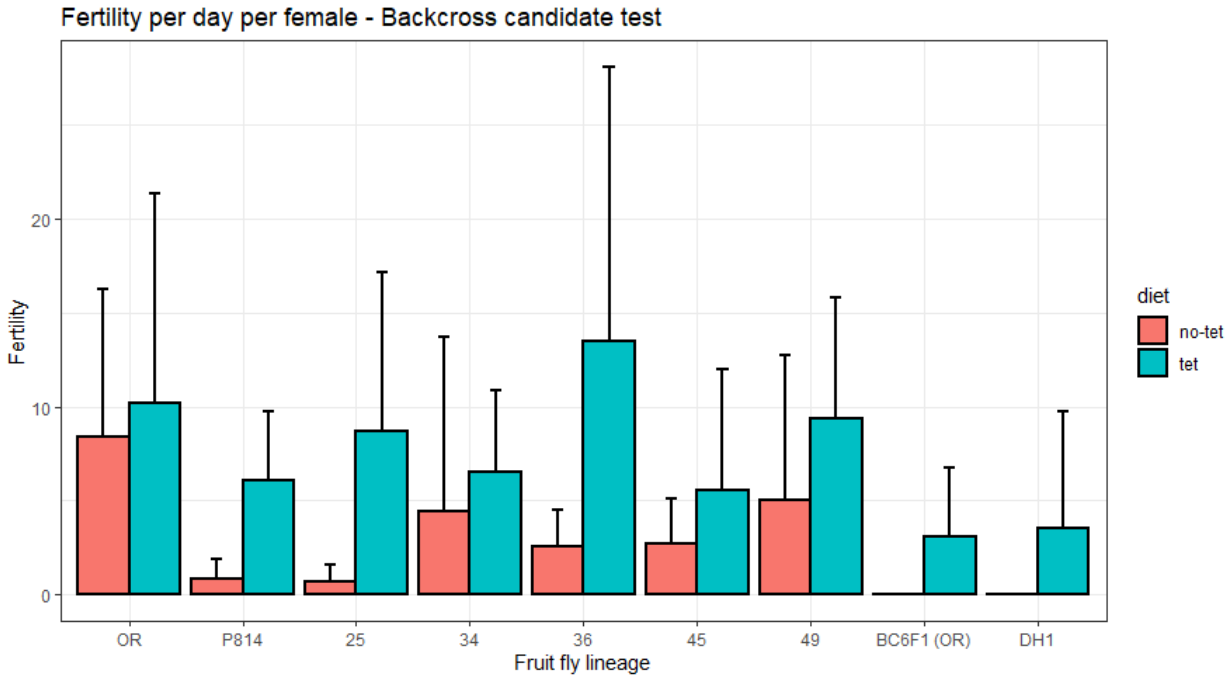

Figure 6 Fertility per day per female of each line in the presence and absence of tetracycline.

Five mating pairs were created for each line, therefore the results for the embryos, larvae, and emergence are showing the number of the 5 lines where presence of each stage was observed. There was one mating pair from line 36 where the data was compromised as the number of surviving adults was unable to be analyzed. Therefore the data for that line should be assessed accordingly, as a ratio out of 4 rather than 5.

Discussion

The results do not support the hypothesis outlined in **Table 1**. There was some growth past the embryonic stage into the larval stage in every line and in both presence and absence of tetracycline. This could be due to several different factors. The first of which is the fact that the parentals for the lines were not drained of the tetracycline for a significant amount of time, thus leaving trace amounts of tetracycline left in their systems and passed on to the progeny. This is a plausible solution as the confirmed double homozygous transgenic lines observed presence of larvae but no emergence. This means the lethal transgene was present, but most likely partially inhibited due to the trace amounts of tetracycline.

As this was the speculation for the reason the DH1 and BC6F2 Oregon R lines did not match the hypothesis, the same could be true for some of the lines from the 5 candidates of BC6F2 P814. However, there were several vials from these candidates that observed some emergence in the absence of tetracycline, which could not have been explained by as the lethal transgene would have hindered the development if not completely, enough to halt development past the larval stage. These lines were most likely not double homozygous. This means one of the markers may have been missing from one of the parents, thus splitting the transgene, losing its effect on the offspring.

The results did, however, prove that the transgenic, native population could be used for effective insect population control. The BC6F2 P814 candidates were more fertile than the P814 lines. All 5 candidates of the BC6F2 P814 line saw higher fertility per day per female than the P814 in both presence and absence of tetracycline. This means the number of females in the BC6F2 P814 line that fostered viable offspring (in the presence of tetracycline) was higher than

for the P814 line. Therefore the BC6F2 P814 line is a high efficiency line and the males could possibly outcompete the native males for mating with the native females.

This conclusion is limited because the experimental crosses of BC6F2 (P814) are only candidates for the selection of a final double homozygous line and are not confirmed double homozygous lines. However, the candidates were those that had the greatest probability of double homozygosity. Therefore, since all 5 of the candidates observed fewer lines with surviving adults than those with the presence of embryos, one of these 5 candidates can be assumed to contain a true double homozygous line. Therefore, the line that is to be declared double homozygous can be assumed to have the same overall result as found by these 5 candidates.

Conclusion

Limitations

The P814 population observed one of the lowest fertility per day per female in the non-tetracycline vials. However, 4 of the 5 lines of P814 had some emergence, meaning each line that produced viable offspring did not produce many. This was not expected because this is the native line that thrives under the right conditions. This means the conditions under which these lines were mating were not ideal. Most likely this is because the parentals for these lines were taken from a previous, fully developed P814 line, and the *Drosophila* that were randomly selected for this experiment may have been older than the parentals of the other lines, therefore past the optimal mating stage. This may skew the results. However, since there were 5 lines

created, and the difference between some of the BC6F2 P814 lines and the P814 lines was significantly large, the results can still be considered valid when determining the difference in fertility of the lines.

There is also a discrepancy between the true and observed results that may lie in the fact that 5 candidates for double homozygosity were used rather than 1 confirmed double homozygous line. However, as described in the discussion section, there is a high probability that one of the candidates contained a double homozygous line, therefore the results can be assumed applicable to a true double homozygous line.

This process was conducted using a line native to Lexington, Kentucky and due to variations in the genome from other populations this process may not be fully applicable to all other species. However, this method may be applicable to some other insect populations such as mosquitos. Research on using a conditionally lethal transgene has been conducted successfully on mosquitos to find positive results (Luke, 2002). The hid gene only exists in the *Drosophila* species, however there are other conditionally lethal genes such as the RIDL gene that suppress production of offspring and can be used for other species (Handler, 2004). Therefore this lethal transgene backcross method for population control may be applicable to varying species, however further research may be necessary.

The lethal transgene may also be more or less effective in different environments. These results were tested in a lab in vials with plentiful food and at 25℃; this is an optimal environment, yet one that differs from the native environment. Therefore when using this lethal transgenic native line for insect population control in a native environment the efficacy of the line, and thus the efficacy of the method of insect population control, may vary.

Implications

Due to the fact that all 5 candidates of BC6F2 P814 observed higher or equal fertility to the other lines, this process of backcrossing a lethal transgene into a native population is a viable option for testing as an insect population control. The applications and extensions of these results are not yet tested, so the full extent of the success of this backcross and the population's mating efficacy cannot be fully determined when in direct competition with other populations. This research is a comparison between populations mating within their own lines. The competition of mating between these populations will need to be tested in order to further determine the applications of this backcross. However, the fertility of the backcrossed native line can be determined through this study to be high enough to be competitive with the native population.

The marker-assisted backcross method has been used and perfected using many varying organisms including rice, mosquitoes, and laboratory grown lines of *Drosophila*. However the applications of this backcross process on native lines has been little studied. This research proves the method could be effective on populations with more genetic variation such as the native lines. The applications for this discovery include the discussed insect population control as well as conducting this backcross on other, more complex species to be able to safely introduce a transgene into a population.

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