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Hybrid Viability: An analysis of Drosophila hybrid competition and mating success amongst its parental species

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Hybrid Viability: An analysis of *Drosophila* hybrid competition and mating success amongst its parental species

By

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Introduction

A primary objective of evolutionary biology centers around the study and analysis of how species form, diverge, and maintain their species-specific differences. Such study is most prevalent during the examination of, relatively speaking, recently diverged species during which modes of selection and species' interactions can shed light on the level(s) of incompatibility that have arisen between the two species. Such is this case with the two species of *Drosophila*, *Drosophila athabasca* (WN - West Northern) and *Drosophila mahican* (EA - Eastern A) which were determined to have diverged from a common ancestor roughly 200k and 100k years ago, respectively. The WN species (*D. athabasca*) occupies a large geographical region across the northern and western portions of North America, whereas EA (*D. mahican)* occupies the North East, thus forming a partial sympatric zone in which both species can be located (**Figure 1)**.

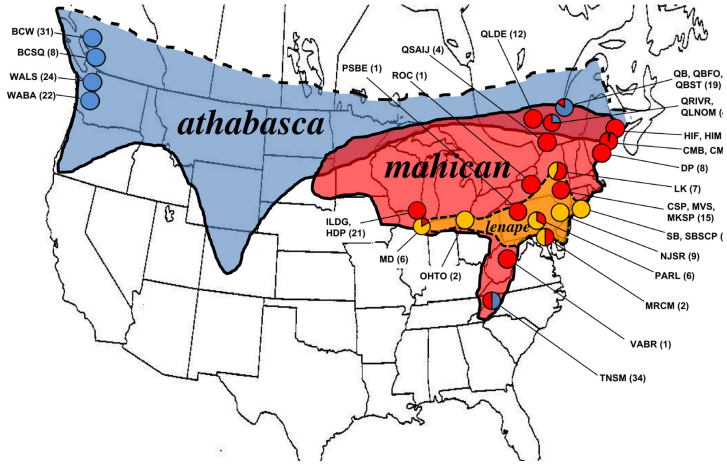


Figure 1. Geographical range map of *Drosophila athabasca* (West Northern) and *Drosophila mahican* (Eastern A) (Miller, 1958)

These two species have been shown to be interfertile despite displaying significant sexual isolation in male courtship song, body size and pigmentation (Sturtevant & Dobzhansky, 1936), and even female mating preferences for conspecifics (Yukilevich et al. 2018). The level of sexual isolation was historically presented by no-choice mating crosses between females and males of the two opposing species in which sexual isolation breakdown could take from 10-30 days (Yoon, 1991). Such a scenario would be highly unlikely in nature, but possible within the laboratory environment. As demonstrated in the past, the utilization of playback song experiments with wingless males displayed that sexual isolation between these two species is solely a product of divergent female mating preferences for conspecific male courtship song (Yukilevich et al. 2018). Thus such playback song experiments between the aforementioned species provide researchers a method for much more successful hybrid generation.

These two endemic species are vital to a greater understanding of speciation, as the traits that have caused speciation in the wild have already been identified, and the aforementioned sympatric zone has provided a vital avenue to study on how distinct methods of speciation played a role in their divergence. For example, Coyne and Orr, have distinguished how speciation occurs differently in allopatric as opposed to sympatric species (Turelli 2015). More specifically, reinforcement has been found to lead towards speciation more rapidly in sympatric species. Reinforcement is the process by which natural selection increases reproductive isolation between species, specifically when the process of hybridization is maladaptive, or the produced hybrids suffer from a lowered fitness. This leads to increases in the sexual isolation between the two species, as the two will avoid mating with a foreign species in order to prevent the formation of maladapted offspring.

While this study does not specifically observe the process of reinforcement, it seeks to examine the viability and competitive ability of the hybrid offspring generated by the WN and EA species. Previous studies have already determined that the hybrid offspring, generated through no-choice matings between WN and EA species, are fertile (Miller & Westphal, 1967). Thus it is vital to identify why hybrid offspring are essentially absent in nature, and whether they suffer in any way as opposed to the two pure species. Using the advantage of a controlled laboratory environment, as opposed to a pure natural setting, we are able to examine uncommon scenarios in which Eastern A, West Northern, and hybrid generations are able to occupy the same spaces. Essentially, this study seeks to examine the viability of hybrid offspring throughout their entire life cycle when placed in an environment with their pure, parental species. (This study defines **pure species** as the two parental species of the hybrids generated for this experiment: **Eastern A and West Northern**)

As previously mentioned, this study will allow for a generalized examination of the viability of hybrid offspring throughout their entire life cycle: first as adult flies, later as larvae through the formation of offspring, then their progression towards and through the pupal stage, and finally again as adult flies in the F1 generation (Fernandez-Morena et al. 2007). Throughout all stages of their life cycle, hybrid offspring will have to compete for food, survive, grow, and attain mates all while placed in an environment alongside the two pure species. Thus this study will not only encompass an examination of a hybrid's ability to survive, but also shed light on any mating preferences between the hybrids or the pure species. By randomly sampling and identifying flies from the F1 generation across numerous replicates of these aforementioned WN, EA and hybrid experimental environments, we will be able to determine by statistical analysis

whether the hybrid flies suffer throughout their life cycle as compared to the pure species (EA, WN). In the case that hybrid flies are not prevalent in the F1 samplings, we will not be able to identify the specific causality behind their suffering from this blanket study. However, pursuing other fly competition trials alongside the combined EA, WN, and hybrid trials (coined as experimental trials within this study) such as “pure species x pure species” control trials, “alternative hybrid x individual pure species” trials, and fecundity trials in which offspring are counted and examined across the three fly species, may elucidate the competitive relationships between the three fly species and their respective levels of sexual isolation.

While male courtship song and female selection upon male courtship song have been shown to be major proponents of sexual isolation between the West Northern and Eastern A species, the mating preferences and ability of hybrids to mate with either pure species are still not completely clear. Furthermore, the viability of hybrids in a competitive setting with the two pure parental species is yet to be discerned. *Drosophila* males are able to vibrate their wings to produce a series of pulses which allow the males to both court females while distinguishing their species identity as courtship song appears quite divergent among the two species (Shirangani et al. 2013). This study will seek to elucidate the ability of the hybrids to mate with either of the pure species.

Therefore this study will seek to discover if hybrid populations produced from WN and EA cross-breedings suffer in any manner when set in a competitive environment with their parental species. This study hypothesizes that the hybrid populations will in fact suffer, or appear at a smaller proportion within the F1 offspring of these competitive bottle experiments as compared to their parental counterparts.

Self-Fertilization Hypothesis

The Self-Fertilization Hypothesis postulates that when sexual reproduction is involved, a very extreme type of inbreeding can occur. Effectively, members of the same populations, or species, will preferentially mate, to an extreme degree, with only their own populations or species, as opposed to outcrossing with any other populations or species. This hypothesis, when applied to this experimental study, would indicate that Eastern A flies would only mate with other Eastern A flies, West Northern would only mate with West Northern, and hybrids would only mate with hybrids. This hypothesis postulates the absence of any out-crossing (i.e. mating with other populations/species). This model implies that the pure parental species would not outcross with each other nor with the hybrid populations, thus expressing a selection against the hybrids, as hybrids will suffer from inbreeding and lose their genotypic frequency. In conclusion, whilst allowing for segregation of alleles in the heterozygote, this hypothesis demonstrates that there is no overall change in allelic frequencies due to self-fertilization, but genotypic proportions are greatly changed due to the regeneration of the two parental species through hybrid-hybrid matings. Only hybrid-hybrid matings greatly alter the genotypic proportions as the pure species matings (EA x EA, WN x WN) result in equal proportions of identical offspring. Hybrid-hybrid matings will result in a halving of heterozygosity and a quarter chance of the regeneration of either pure species (**Figure 2)**. Finally, this hypothesis postulates that no hybrids are regenerated through pure species outcrossings (EA x WN).

♂\♀	A	a
A	AA	aA
a	Aa	aa

Figure 2. Mendeleian monohybrid cross. Due to the Mendeleian monohybrid cross which would occur in the self-fertilization model, the resulting offspring of hybrid-hybrid matings should display genotypes of AA, Aa, Aa, and aa, or potential phenotypes of 25% WN, 50% hybrid, and 25% EA, greatly altering the genotypic proportions of the succeeding generation.

Partial Self-Fertilization Hypothesis

The Partial Self-Fertilization Hypothesis postulates that when sexual reproduction is involved, a certain degree of outcrossing (random mating) can occur. Effectively, members of the same population or species can randomly mate with other populations or species within the same environment, alongside mating within their own populations or species. This hypothesis develops upon the idea of self-fertilization by also accounting for those individuals within a population who chose to mate outside of their own populations/species, therefore producing different genotypic frequencies in the next generation of offspring. This model implies that there would be no selection against the hybrid population as all populations in the experimental trials would openly outcross with each other. Essentially, this model would imply that reinforcement does not occur within these micro-environments between the two pure species, and that sexual isolation would actually be minimized. Once again, while individual allelic frequencies do not change, genotypic frequencies can change over time. Unlike in complete self-fertilization, partial self-fertilization allows for an outcrossing of the pure species (EA x WN) thereby regenerating the hybrid population, while also allowing the hybrid population to outcross with either of the pure species **(Figures 3a, 3b)**. Thus, this hypothesis concludes that in a scenario in which inbreeding (self-fertilization) and outcrossing (random mating) occur in equal proportions, the next generation should feature genotypic frequencies representative of the parental generation.

♂\♀	A	A
a	Aa	Aa
a	Aa	Aa

Figure 3a. Mendelian Pure Species Outcross. Due to the Mendeleian homozygous outcross which would occur within the partial self-fertilization model, the resulting matings of such outcrosses would allow for the total regeneration of the hybrid genotype. 100% of the offspring of such crosses would consist of the Aa hybrid genotype. Other than the hybrid-hybrid self-fertilization mating type, the homozygous outcross is the only other mating type which allows for any regeneration of the hybrid population.

♂\♀	A	A
A	AA	AA
a	Aa	Aa

Figure 3b. Mendelian Hybrid and Pure Species Outcross. Due to the Mendeleian hybrid and homozygous pure species outcross which would occur within the partial self-fertilization model, the resulting matings of such outcrosses would allow for a preservation of hybrid and respective pure species genotypes.

Methodology

Model Calculations

P_1 - Genotypic Frequency of F1 Pure Species #1 (F1 EA)

H_1 - Genotypic Frequency of F1 Hybrids (Hybrids)

Q_1 - Genotypic Frequency of F1 Pure Species #2 (F1 WN)

P_0 - Genotypic Frequency of Parental Generation Pure Species #1 (EA)

H_0 - Genotypic Frequency of Parental Generation Hybrids (Hybrid)

Q_0 - Genotypic Frequency of Parental Generation Pure Species #2 (WN)

$$P_1 = P_0 + \tfrac{1}{4} H_0 \quad (1)$$

$$H_1 = \tfrac{1}{2} H_0 \quad (2)$$

$$Q_1 = Q_0 + \tfrac{1}{4} H_0 \quad (3)$$

Equations 1-3 and associated Equation Variable Denotation Table. Equations 1-3 represent the equations utilized in the calculation of genotypic frequencies under the **Self-Fertilization Model**. (Hedrick, Genetics of populations)

P_1 - Genotypic Frequency of F1 Pure Species #1 (F1 EA)

H_1 - Genotypic Frequency of F1 Hybrids (Hybrids)

Q_1 - Genotypic Frequency of F1 Pure Species #2 (F1 WN)

P_0 - Genotypic Frequency of Parental Generation Pure Species #1 (EA)

H_0 - Genotypic Frequency of Parental Generation Hybrids (Hybrid)

Q_0 - Genotypic Frequency of Parental Generation Pure Species #2 (WN)

S - proportion of progeny produced by self-fertilization

T - proportion of progeny produced by outcrossing

p_0 - initial allelic frequency of A_1 (EA - A_1A_1)

q_0 - initial allelic frequency of A_2 (WN - A_2A_2)

$$P_1 = T(p_0)^2 + S(P_0 + \tfrac{1}{4}H_0) \qquad (4)$$

$$H_1 = 2T(p_0)(q_0) + \tfrac{1}{2}(S)(H_0) \qquad (5)$$

$$Q_1 = T(q_0)^2 + S(Q_0 + \tfrac{1}{4}H_0) \qquad (6)$$

Equations 4-6 and associated Equation Variable Denotation Table. Equations 4-6 represent the equations utilized in the calculation of genotypic frequencies under the **Partial Self-Fertilization Model**. (Hedrick, Genetics of populations)

Experimental Trial Calculations

Self-Fertilization Model Calculations (Hedrick, Genetics of populations)

Equations 1-3 are utilized in the calculation of prospective F1 genotypic frequencies of the EA, hybrid and WN species, respectively, specifically in the experimental trials involving the three aforementioned species. As each species/population is present within equal proportions within each experimental trial, the respective initial genotypic frequencies for each genotype categorization are ⅓, or 0.33 (see **Table 1** for calculated frequencies).

Partial Self-Fertilization Model Calculations (Hedrick, Genetics of populations)

Equations 4-6 are utilized in the calculation of prospective F1 genotypic frequencies of the EA, hybrid and WN species, respectively, specifically in the experimental trials involving the three aforementioned species. As each species/population is present within equal proportions within each experimental trial, the respective initial genotypic frequencies for each genotype categorization are ⅓, or 0.33. Furthermore, the initial allelic frequencies **(p_0 and q_0)** are equivalent and set to values of 0.5. The partial self-fertilization model asserts the use of the variable S, to represent proportion of the progeny produced by self-fertilization, and the use of the variable T, to present the proportion produced by outcrossing, such that $S + T = 1$. Effectively, by utilizing values of 0.5 for both S and T variables, the partial self-fertilization model will describe a scenario wherein random mating is prevalent along with self-fertilization (see **Table 1** for calculated frequencies).

Experimental Trial Model Frequencies **(See Figures 4, 5 for Calculations)**

Genotypic Designation	**Species**	**Self-Fertilization F1 Genotypic Frequency**	**Partial Self-Fertilization F1 Genotypic Frequency**
P_1	**EA**	**0.4125**	**0.33125**
H_1	**Hybrid**	**0.165**	**0.3325**
Q_1	**WN**	**0.4125**	**0.33125**

Table 1. Experimental Trial: Calculated F1 Genotypic Frequencies for both models.

Pure Species Control Calculations

Self-Fertilization Model Calculations

Equations 1-3 are utilized in the calculation of prospective F1 genotypic frequencies of the EA x WN Pure Species Control Replicates. More specifically, these control trials will only feature the presence of both pure species in equal proportions. Thus, the initial genotypic frequency of EA (P_0) and the initial genotypic frequency of WN (Q_0) will be set as 0.5. With no hybrids present within the Pure Species Control Replicates, the initial genotypic frequency of the hybrid population (H_0) will be set as 0.0 (see **Table 2** for calculated frequencies).

Partial Self-Fertilization Model Calculations

Equations 4-6 are utilized in the calculation of prospective F1 genotypic frequencies of the Pure Species Control Replicates, in which only the Eastern A and West Northern Fly Types are present, in equal proportion. Thus, with the absence of a hybrid population within these trials, the initial genotypic frequencies of EA and WN are set to 0.5. Furthermore, the initial allelic frequencies **(p_0 and q_0)** are equivalent and set to values of 0.5. Yet again, the partial self-fertilization model asserts the use of the variable S, to represent the proportion of the progeny produced by self-fertilization, and the use of the variable T, to present the proportion produced by outcrossing, such that S + T = 1. Effectively, by utilizing values of 0.5 for both S and T variables, the partial self-fertilization model will describe a scenario wherein random mating and thus outcrossing is prevalent along with self-fertilization (see **Table 2** for calculated frequencies).

Pure Species Control Model Frequencies **(See Figures 6, 7 for Calculations)**

Genotypic Designation	**Species**	**Self-Fertilization F1 Genotypic Frequency**	**Partial Self-Fertilization F1 Genotypic Frequency**
P_1	**EA**	**0.5**	**0.375**
H_1	**Hybrid**	**0.0**	**0.25**
Q_1	**WN**	**0.5**	**0.375**

Table 2. Pure Species Control Trial: Calculated F1 Genotypic Frequencies for both models.

Alternative Hybrid x Individual Pure Species Calculations

Self-Fertilization Model Calculations for each Alternate Combination

Equations 1-3 are utilized in the calculation of prospective F1 genotypic frequencies of the Hybrid x Individual Pure Species "Alternate Replicates" according to the Self-Fertilization Model. More specifically, these trials will feature the presence of one pure species, EA or WN, in equal proportion to a hybrid population. Hybrid populations are divided into two separate factions: Hybrids born to West Northern mothers, and Hybrids born to Eastern A mothers. This distinction is made as the male courtship song appears to be linked to the X chromosome and thus determined by the identity of the mother. These trials will help establish the competitive and mating relationships between the above specified hybrids and one of the two pure species. Thus, the following pages will describe the variety of combinations of calculations related to which pure species is being tested against which hybrid type. Each fly type, whether hybrid or pure species, will be present in equal proportion in each alternate trial, thus offering a value of 0.5 for each initial genotypic frequency (see **Tables 3-6** for calculated frequencies).

Partial Self-Fertilization Model Calculations

Equations 4-6 are utilized in the calculation of prospective F1 genotypic frequencies of the Hybrid x Individual Pure Species "Alternate Replicates" according to the Partial Self-Fertilization Model. More specifically, these trials will feature the presence of one pure species, EA or WN, in equal proportion to a hybrid population. Hybrid populations are divided into two separate factions: Hybrids born to West Northern mothers, and Hybrids born to Eastern A mothers. This distinction is made as the male courtship song appears to be linked to the X

chromosome and thus determined by the identity of the mother. These trials will help establish the competitive and mating relationships between the above specified hybrids and one of the two pure species while involving the ability of the flies to outcross between each other. Thus, the following pages will describe the variety of combinations of calculations related to which pure species is being tested against which hybrid type. Each fly type, whether hybrid or pure species, will be present in equal proportion in each alternate trial, thus offering a value of 0.5 for each initial genotypic frequency. Yet again, the partial self-fertilization model asserts the use of the variable S, to represent the proportion of the progeny produced by self-fertilization, and the use of the variable T, to present the proportion produced by outcrossing, such that $S + T = 1$. Effectively, by utilizing values of 0.5 for both S and T variables, the partial self-fertilization model will describe a scenario wherein random mating and thus outcrossing is prevalent along with self-fertilization. However, the values of p_0 and q_0 will vary across the alternate trials, as these trials deal with one population of hybrids and one population of pure species. Thus the respective initial allelic values will not both be equivalent to 0.5 within these calculations (see **Tables 3-6** for calculated frequencies).

Alternate Trial Model Frequencies **(See Figures 8, 9 for Calculations)**

EA Pure (P_0) x Hybrids derived from WN Mothers (H_0)

Genotypic Designation	Species	Self-Fertilization F1 Genotypic Frequency	Partial Self-Fertilization F1 Genotypic Frequency
P_1	EA	0.625	0.594
H_1	Hybrid	0.25	0.3125
Q_1	WN	0.125	0.09375

Table 3. Alternate Trial EA mix x WNDH: Calculated F1 Genotypic Frequencies for both models.

EA Pure (P_0) x Hybrids derived from EA Mothers (H_0)

Genotypic Designation	Species	Self-Fertilization F1 Genotypic Frequency	Partial Self-Fertilization F1 Genotypic Frequency
P_1	EA	0.625	0.594
H_1	Hybrid	0.25	0.3125
Q_1	WN	0.125	0.09375

Table 4. Alternate Trial EA mix x EADH: Calculated F1 Genotypic Frequencies for both models.

WN Pure (Q_0)x Hybrids derived from WN Mothers (H_0)

Genotypic Designation	Species	Self-Fertilization F1 Genotypic Frequency	Partial Self-Fertilization F1 Genotypic Frequency
P_1	EA	0.125	0.09375
H_1	Hybrid	0.25	0.3125
Q_1	WN	0.625	0.594

Table 5. Alternate Trial WN mix x WNDH: Calculated F1 Genotypic Frequencies for both models.

WN Pure (Q_0) x Hybrids derived from EA Mothers (H_0)

Genotypic Designation	Species	Self-Fertilization F1 Genotypic Frequency	Partial Self-Fertilization F1 Genotypic Frequency
P_1	EA	0.125	0.09375
H_1	Hybrid	0.25	0.3125
Q_1	WN	0.625	0.594

Table 6. Alternate Trial WN mix x EADH: Calculated F1 Genotypic Frequencies for both models.

Raw Calculations

$P_0 = 0.33, H_0 = 0.33, Q_0 = 0.33$

$P_1 = P_0 + ¼ H_0 = (0.33) + ¼ (0.33)$ -- $P_1 = \underline{0.4125}$

$H_1 = ½ H_0 = ½ (0.33)$ -- $H_1 = \underline{0.165}$

$Q_1 = Q_0 + ¼ H_0 = (0.33) + ¼ (0.33) = \underline{0.4125}$

Figure 4. Raw Experimental Trial, Self-Fertilization Model Calculations and Variable Designations.

$P_0 = 0.33, H_0 = 0.33, Q_0 = 0.33, p_0 = 0.5, q_0 = 0.5, S = 0.5, T = 0.5$

$P_1 = T(p_0)^2 + S(P_0 + ¼H_0) = (0.5)(0.5)^2 + (0.5)(0.33 + ¼(0.33))$ -- $P_1 = \underline{0.33125}$

$H_1 = 2T(p_0)(q_0) + ½(S)(H_0) = (2 * 0.5)(0.5)(0.5) + ½(0.5)(0.33)$ -- $H_1 = \underline{0.3325}$

$Q_1 = T(q_0)^2 + S(Q_0 + ¼H_0) = (0.5)(0.5)^2 + (0.5)(0.33 + ¼(0.33))$ -- $Q_1 = \underline{0.33125}$

Figure 5. Raw Experimental Trial, Partial Self-Fertilization Model Calculations and Variable Designations.

$P_0 = 0.5, H_0 = 0.0, Q_0 = 0.5$

$P_1 = P_0 + ¼ H_0 = 0.5 + ¼ (0)$ -- $P_1 = \underline{0.5}$

$H_1 = ½ H_0 = ½ (0)$ -- $H_1 = \underline{0.0}$

$Q_1 = Q_0 + ¼ H_0 = 0.5 + ¼ (0)$ -- $Q_1 = \underline{0.5}$

Figure 6. Raw Pure Species Control Trial, Self-Fertilization Model Calculations and Variable Designations.

$P_0 = 0.5, H_0 = 0.0, Q_0 = 0.5, p_0 = 0.5, q_0 = 0.5, S = 0.5, T = 0.5$

$P_1 = T(p_0)^2 + S(P_0 + ¼H_0) = (0.5)(0.5)^2 + (0.5)(0.5 + ¼(0))$ -- $P_1 = \underline{0.375}$

$H_1 = 2T(p_0)(q_0) + ½(S)(H_0) = (2)(0.5)(0.5)(0.5) + ½(0.5)(0)$ -- $H_1 = \underline{0.25}$

$Q_1 = T(q_0)^2 + S(Q_0 + ¼H_0) = (0.5)(0.5)^2 + (0.5)(0.5 + ¼(0))$ -- $Q_1 = \underline{0.375}$

Figure 7. Raw Pure Species Control Trial, Partial Self-Fertilization Model Calculations and Variable Designations.

EA Pure (P_0) x Hybrids derived from WN Mothers (H_0)

$P_0 = 0.5, H_0 = 0.5, Q_0 = 0.0$

$P_1 = P_0 + ¼ H_0 = 0.5 + ¼ (0.5)$ -- $P_1 = \underline{0.625}$

$H_1 = ½ H_0 = ½ (0.5)$ -- $H_1 = \underline{0.25}$

$Q_1 = Q_0 + ¼ H_0 = 0 + ¼ (0.5)$ -- $Q_1 = \underline{0.125}$

EA Pure (P_0) x Hybrids derived from EA Mothers (H_0)

$P_0 = 0.5, H_0 = 0.5, Q_0 = 0.0$

$P_1 = P_0 + ¼ H_0 = 0.5 + ¼ (0.5)$ -- $P_1 = \underline{0.625}$

$H_1 = ½ H_0 = ½ (0.5)$ -- $H_1 = \underline{0.25}$

$Q_1 = Q_0 + ¼ H_0 = 0 + ¼ (0.5)$ -- $Q_1 = \underline{0.125}$

WN Pure (Q_0)x Hybrids derived from WN Mothers (H_0)

$P_0 = 0.0, H_0 = 0.5, Q_0 = 0.5$

$P_1 = P_0 + ¼ H_0 = 0 + ¼ (0.5)$ -- $P_1 = \underline{0.125}$

$H_1 = ½ H_0 = ½ (0.5)$ -- $H_1 = \underline{0.25}$

$Q_1 = Q_0 + ¼ H_0 = 0.5 + ¼ (0.5) = \underline{0.625}$

WN Pure (Q_0) x Hybrids derived from EA Mothers (H_0)

$P_0 = 0.0, H_0 = 0.5, Q_0 = 0.5$

$P_1 = P_0 + ¼ H_0 = 0 + ¼ (0.5)$ -- $P_1 = \underline{0.125}$

$H_1 = ½ H_0 = ½ (0.5)$ -- $H_1 = \underline{0.25}$

$Q_1 = Q_0 + ¼ H_0 = 0.5 + ¼ (0.5) = \underline{0.625}$

Figure 8. Raw Alternate Trial, Self-Fertilization Model Calculations and Variable Designations.

EA Pure (P_0) x Hybrids derived from WN Mothers (H_0)

$P_0 = 0.5, H_0 = 0.5, Q_0 = 0.0, p_0 = 0.75, q_0 = 0.25, S = 0.5, T = 0.5$

$P_1 = T(p_0)^2 + S(P_0 + \frac{1}{4}H_0) = (0.5)(0.75)^2 + (0.5)(0.5 + \frac{1}{4}(0.5))$ -- $P_1 = \underline{0.594}$

$H_1 = 2T(p_0)(q_0) + \frac{1}{2}(S)(H_0) = (2)(0.5)(0.75)(0.25) + \frac{1}{2}(0.5)(0.5)$ -- $H_1 = \underline{0.3125}$

$Q_1 = T(q_0)^2 + S(Q_0 + \frac{1}{4}H_0) = (0.5)(0.25)^2 + (0.5)(0 + \frac{1}{4}(0.5))$ -- $Q_1 = \underline{0.09375}$

EA Pure (P_0) x Hybrids derived from EA Mothers (H_0)

$P_0 = 0.5, H_0 = 0.5, Q_0 = 0.0, p_0 = 0.75, q_0 = 0.25, S = 0.5, T = 0.5$

$P_1 = T(p_0)^2 + S(P_0 + \frac{1}{4}H_0) = (0.5)(0.75)^2 + (0.5)(0.5 + \frac{1}{4}(0.5))$ -- $P_1 = \underline{0.594}$

$H_1 = 2T(p_0)(q_0) + \frac{1}{2}(S)(H_0) = (2)(0.5)(0.75)(0.25) + \frac{1}{2}(0.5)(0.5)$ -- $H_1 = \underline{0.3125}$

$Q_1 = T(q_0)^2 + S(Q_0 + \frac{1}{4}H_0) = (0.5)(0.25)^2 + (0.5)(0 + \frac{1}{4}(0.5))$ -- $Q_1 = \underline{0.09375}$

WN Pure (Q_0)x Hybrids derived from WN Mothers (H_0)

$P_0 = 0.0, H_0 = 0.5, Q_0 = 0.5, p_0 = 0.25, q_0 = 0.75, S = 0.5, T = 0.5$

$P_1 = T(p_0)^2 + S(P_0 + \frac{1}{4}H_0) = (0.5)(0.25)^2 + (0.5)(0 + \frac{1}{4}(0.5))$ -- $P_1 = \underline{0.09375}$

$H_1 = 2T(p_0)(q_0) + \frac{1}{2}(S)(H_0) = (2)(0.5)(0.25)(0.75) + \frac{1}{2}(0.5)(0.5)$ -- $H_1 = \underline{0.3125}$

$Q_1 = T(q_0)^2 + S(Q_0 + \frac{1}{4}H_0) = (0.5)(0.75)^2 + (0.5)(0.5 + \frac{1}{4}(0.5))$ -- $Q_1 = \underline{0.594}$

WN Pure (Q_0) x Hybrids derived from EA Mothers (H_0)

$P_0 = 0.0, H_0 = 0.5, Q_0 = 0.5, p_0 = 0.25, q_0 = 0.75, S = 0.5, T = 0.5$

$P_1 = T(p_0)^2 + S(P_0 + \frac{1}{4}H_0) = (0.5)(0.25)^2 + (0.5)(0 + \frac{1}{4}(0.5))$ -- $P_1 = \underline{0.09375}$

$H_1 = 2T(p_0)(q_0) + \frac{1}{2}(S)(H_0) = (2)(0.5)(0.25)(0.75) + \frac{1}{2}(0.5)(0.5)$ -- $H_1 = \underline{0.3125}$

$Q_1 = T(q_0)^2 + S(Q_0 + \frac{1}{4}H_0) = (0.5)(0.75)^2 + (0.5)(0.5 + \frac{1}{4}(0.5))$ -- $Q_1 = \underline{0.594}$

Figure 9. Raw Alternate Trial, Partial Self-Fertilization Model Calculations and Variable Designations.

Fly Preparation

This experiment utilized mixed lines of both Eastern A and West Northern *Drosophila*. The generation of hybrids was undertaken in two concurrent phases, in which 15-20 males and females of opposite species were placed in a chamber for approximately 15 minutes. The first phase consisted of EA females and WN males, while the second consisted of WN females and EA males. All flies utilized within the hybrid generation trials were collected soon after birth to ensure virginity before the trials. Mating trials were conducted in the mornings with the flies kept in the dark until the commencement of a generation trial. As EA and WN flies are sexually isolated and mate identification depends on the species-specific male wing flap pattern, audio playback of the corresponding female's species wing flap pattern was played via a speaker into the mating chamber. Thus during the phase in which female EA flies mated with WN males, audio playback of the EA male wing pattern song was played through the speaker in order to mask the WN male song and allow for successful mating between the two separate species. The opposite scenario was applied to the phase of WN female flies. Both phases featured a mixture of replicate trials in which male flies had their wings intact with the speaker drowning out their corresponding wing flap patterns, or other replicates in which the males were wingless paired with the audio playback. Any mating pairs of flies were extracted out of the mating chamber via a fly aspirator and were placed into fresh food vials. The flies were then transferred every three days into new food vials to maximize potential egg laying. Later, the hybrid offspring were collected and separated according to their sex and were aged for a period of time ranging from one week to ten days.

Bottle Organization

_____Bottles were utilized as experimental chambers for the competition experiment. Each bottle consisted of 4 cups of dry fly medium mixed with 2 full narrow vials of distilled water. Bottles were spun by hand in circular motions if necessary, in order to allow for the settlement of food at the bottom of the chamber and to ensure a complete mixing with the water. After the food had dried and solidified, four small strips of paper were folded individually and placed in a cross pattern within the center of each bottle, nestled in the upper most layer of the food. 20 individual pellets of yeast were then dropped into each bottle. The bottle experiments were conducted within two separate phases, one week apart. This was to ensure that there were enough flies collected and properly aged for sexual reproduction as virgin fly yield was not always consistent. Upon initial setup, flies were organized by age and randomized across trials so as to not create age distinctions within the trials with younger/older flies only within certain replicates. The first bottle phase contained 19 bottles, while the second phase conducted a week later involved 22 bottles, for a total of 41 bottles. Each bottle, regardless of categorization or purpose, was initially filled with 60 flies.

Bottle trials were organized into four separate categories:

1. **Experimental** - Experimental bottle types involved a pure competitive environment for the two pure species (WN, EA) and hybrids. 10 EA males, 10 EA females, 10 WN females, and 10 WN males made up the pure species' placed into the bottle. The hybrids were divided into two separate groups, involving 5 hybrid males and 5 hybrid females born to EA mothers, with the other ten hybrids consisting of 5 hybrid males and 5 hybrid females born to WN mothers.

2. **Pure Species** - Pure Species bottle types involved a pure competitive environment for the two parental species alone (WN, EA). 15 EA males, 15 EA females, 15 WN males, and 15 WN females were introduced into each *Pure Species* bottle. This was to assess whether one species would have any edge over the other in terms of offspring production or environmental ability.
3. **Fecundity Controls** - Fecundity control bottles contained only one species type (either, WN, EA or hybrids) which served to assess any differences in fecundity between the three fly types.
4. **Hybrid Alternates** - Hybrid alternate bottles involve the placement of 30 hybrid flies with 30 flies of one of the pure species. The hybrids were divided into two subdivisions: those hybrids born to EA mothers, and those born to WN mothers. This is because male courtship song appears to be not only autosomally linked, but also X-linked, and thus the specific song expressed by the male appears to be linked to the species' identity of the mother. These trials served to assess whether there were differences in preference of a pure species towards the hybrids while further demonstrating potential competitive distinctions between the hybrids and pure species (Yukilevich et al. 2016).

Before the commencement of the bottle phase, all flies were organized into empty narrow vials depending on their species, sex, and age, and placed into vial clusters in preparation for placement into their respective bottle type(s). As previously mentioned, the vials were shuffled among the clusters, however, to ensure randomness of age among each bottle. Then all the individual fly vials were temporarily bumped together into one vial before being funneled into the bottle to prevent fly escape. Initial humidity and temperature, as well as the time of commencement were recorded for the two separate bottle phases. Five days after placing the flies

into the bottles, all flies were removed by inoculating them with CO2 while inverting the bottles to prevent any flies from remaining in the bottles. After approximately 10 days post-clearing of the flies, the date of emergence of any offspring flies was noted, and after a three day waiting period, offspring F1 flies were then inoculated with CO2, collected on a plate, and separated by sex. This was to prevent sexual maturation of the flies and the onset of mating, while allowing each species flies to develop. Then, 30 male flies and 30 female flies were identified and randomly selected from each bottle and placed into 1.5 mL eppendorf tubes, separated by sex. Such selection of 60 flies total, and segregation by sex were performed for each bottle of each phase. However, no flies were collected from **Fecundity Control** replicate bottles. Rather a total count of both male and female offspring flies present within those bottles after the three day window was conducted. After a complete collection of the required 30 male and 30 female flies for each bottle was concluded, all eppendorf tubes full of flies were organized by bottle number and sex and placed within a -80 Degree Celsius freezer until stage 3, DNA Extraction.

Bottle Phase	# of Bottles	Date of Fly Placement in Bottles	Date of Fly Clearing from Bottles	Date of Offspring Collection
#1	19	12/11/2020	12/16/2020	12/29/2020
#2	22	12/18/2020	12/23/2020	01/03/2021

Table 7. Bottle Trial Designations, and Dates of Action.

DNA Sequencing

In order to properly identify the flies collected from any bottle, genetic testing of each fly marked the next stage of this experiment.

A. DNA Extraction

The first step of fly DNA extraction involved keeping the flies in a -80 Degree Celsius Freezer. Due to the large amount of samples to be extracted, only female flies were utilized in DNA sequencing. Furthermore, sequencing only occurred for the female flies from 16 different bottles. With 30 females per each bottle, a total of 480 flies were individually sequenced.

The following outlines the protocol performed for the DNA extraction of 30 flies at a time.

DNA Extraction took place with 30 female flies at a time. Pestles for grinding down the flies were all sanitized before the start of the extraction phase, autoclaved, and stored frozen, in the -80 Degrees Celsius freezer along with the flies. A pestle gun was utilized alongside the pestles to enhance the grinding efficiency. It should be noted that clean and sanitized pestles, and pipette tips were utilized for each step of this experimental stage to ensure the lowest levels of sample contamination.

30, 1.5 mL Eppendorf tubes were filled with 100 uL of Cell Lysis Solution and placed within a large ice bucket. These tubes were kept on ice for approximately 15 minutes until the solutions turned cloudy. Then 30 more 1.5 mL Eppendorf tubes were filled with 100 uL of

isopropanol, placed on a tray, and stored in a -20 Degrees Celsius freezer for a later step. Each Eppendorf tube of both Cell Lysis and isopropanol were accordingly labeled with a matching serial number denoting the female sex, and bottle from which the fly had been collected.

Once the Cell Lysis Solutions turned cloudy, a tube of 30 female flies was removed from the -80 Degrees Celsius Freezer and one female was transferred into each of the 30 Cell Lysis tubes. These tubes were kept on ice to ensure the coldest possible temperature of the solution and fly. Once the flies were transferred into the Cell Lysis tubes, grinding of each fly commenced. The aforementioned frozen pestles were removed from the -80 Degree Celsius freezer and attached to a pestle gun. Each fly was grinded for approximately 30 seconds or until no visibly large fragments of the fly were present, or until a distinct color change in the solution was noted. It should be noted that each fly received a distinct pestle for the grinding step, to ensure no contamination of each sample.

Upon the completion of grinding a fly, the foaming of the Cell Lysis was later curbed by conducting a short spin of all 30 Cell Lysis tubes in a centrifuge. All 30 cell lysis and ground fly solutions were then capped and placed in a 65℃ incubator tank for 15 minutes. During this time, RNase A Solution was retrieved from a refrigerator and prepared for the next step.
All 30 cell lysis and ground fly solutions were then uncapped and 0.5 uL of RNase A Solution was pipetted into each of the 30 Eppendorff tubes and placed on an mixing plate for inversion for approximately 2 minutes. Once the tubes were finished inverting, all 30 samples were centrifuged in a short run to rid the samples of bubbles.

All 30 samples were then capped again and placed in a 37℃ incubator for a time period ranging from 15 minutes to an hour. Next, they were removed from the incubator and quickly cooled in an ice bucket. Next 33 uL of Protein Precipitation Solution were added into each of the

30 samples before vortexing each sample vigorously for 20 seconds at the maximum speed. Afterwards, each sample was again placed in the ice bucket for approximately 5 minutes.

Next all 30 samples were centrifuged at 14,000xg for 3 minutes. While the samples were spinning, the tray of 30 marked isopropanol tubes were removed from the -20℃ freezer. By the conclusion of this step, all 30 cell lysis tubes in the centrifuge had a tight pellet at the bottom of their tube. As previously mentioned each cell lysis tube also had an according tube of 100uL of isopropanol. Now, we were able to pour the supernatant (liquid only) from the centrifuged cell lysis tubes into the new isopropanol tubes. The now empty cell lysis tubes (with a pellet) were then discarded.

The new isopropanol and supernatant tubes were placed on the mixing plate and inverted for approximately five minutes. Then each sample was centrifuged at 14,000xg for five minutes. Now the isopropanol tubes had a small white DNA pellet present at the bottom of their tubes. Paper towels and absorbent paper were set up on the lab bench, and this time the supernatant was drained away onto the pieces of paper while the white DNA pellet stayed in the bottom of the tube. After approximately 15 minutes of drying, a bottle of 70% ethanol was removed from the -20℃ freezer, and 100 uL of 70% ethanol was added into each of the dried tubes. All of the samples now filled with 70% ethanol were inverted on the mixing plate for approximately 2 minutes, before being centrifuged a final time at 14,000xg for two minutes.

New paper towels and pieces of absorbent paper were placed on the lab bench. Yet again, the supernatant of each of the samples was drained onto the paper and left to dry. Once the tubes were completely dry, 15 uL of RNase-free water was added to the DNA pellet. All 30 labeled sample tubes were then placed into the -20℃ Freezer overnight.

B. Nano-Drop, PCR

The 30 samples now containing dissolved DNA within the 15 uL of RNase-free water were then removed from the -20℃ freezer, warmed until of liquid consistency, vortexed, and finally short-ran through the centrifuge. Next, each sample was analyzed using a Nano-Drop. The Nano-Drop sensor was initially blanked with RNase-free water and wiped off with diluted water after each sample analysis. 1 uL of each sample was analyzed, and the samples according ng/uL, and 260/280 values were recorded in a table.

After recording the Nano-Drop values, a PCR set-up was performed for each sample. The PCR mix consisted of 10 uL of PCR Master, 7 uL of RNase-free water, 1 uL of the NonA forward primer, 1 uL of the NonA reverse primer, and finally 1 uL of the dissolved DNA sample. Thus, 30 such PCR tubes were created accordingly with the 30 DNA samples.

C. Gel and Exo-Sap

For imaging the PCR products, 1% Agarose gels were prepared within the lab. 1 gram of Agarose was mixed in 100 mL of 1x LB buffer in an Erlenmeyer flask. The flask was then microwaved until the solution began to bubble, and all of the Agarose had dissolved within the solution. The flask was removed from the microwave and let to cool on the lab bench, before being poured into two separate mini-gel molds. The mini-gels allowed the lab to run up to 22 individual samples at a time on a single gel, with the placement of 12-well molds at the top, and the middle of the mini-gels (the left-most well was reserved for the placement of a 1kB ladder within both of the molds).

On one sheet of parafilm, 12, 6 uL droplets of RNase free water were pipetted and placed apart from each other. To each of the 12 drops, 2 uL of EZ dye were added. Finally, one droplet of water/dye received 2 uL of the 1kB ladder, while the other eleven droplets of water/dye received 2 uL of the PCR sample products. Then using a 10 uL pipette, each of the top twelve wells were loaded from left to right with the ladder, and 11 DNA samples, respectively. The same method was repeated for the middle mold of 12 wells. The entire process was then repeated for the second mini-gel, allowing the lab to run two gels at a time, with 44 DNA samples total (as previously described with utilizing this method, 48 available wells, with 4 wells reserved for the 1kB ladder, left room for 44 DNA samples).

Both gels were placed in their own gel boxes, which had been filled with 1x LB Buffer and were connected to the same power source and powered in tandem. The gels were powered at 165 volts, and were powered off when the EZ dye approximately reached half-way across the gel. The gels were then removed from the gel boxes and imaged. Any samples that did not present a band were noted. *At first, all samples regardless of the presence of a band were then processed through an Exo-Sap procedure and sent off for sequencing. However, later on in the experiment, samples without bands in their gel images were omitted from the Exo-Sap procedure and were not sent for sequencing to save costs (See **Figure 4** for sample gel product).

Samples that did present a band then proceeded through an Exo-Sap step. PCR tubes were labelled with serial numbers, and 5 uL of PCR product were placed into each PCR tube. Then 2 uL of Exosap were added into each PCR tube. The Exosap had been kept in the -20℃ freezer for storage, and was kept in an ice bucket throughout its use in this step. Each PCR tube was centrifuged down after the addition of the Exosap, and was placed in the PCR machine for the Exosap procedure.

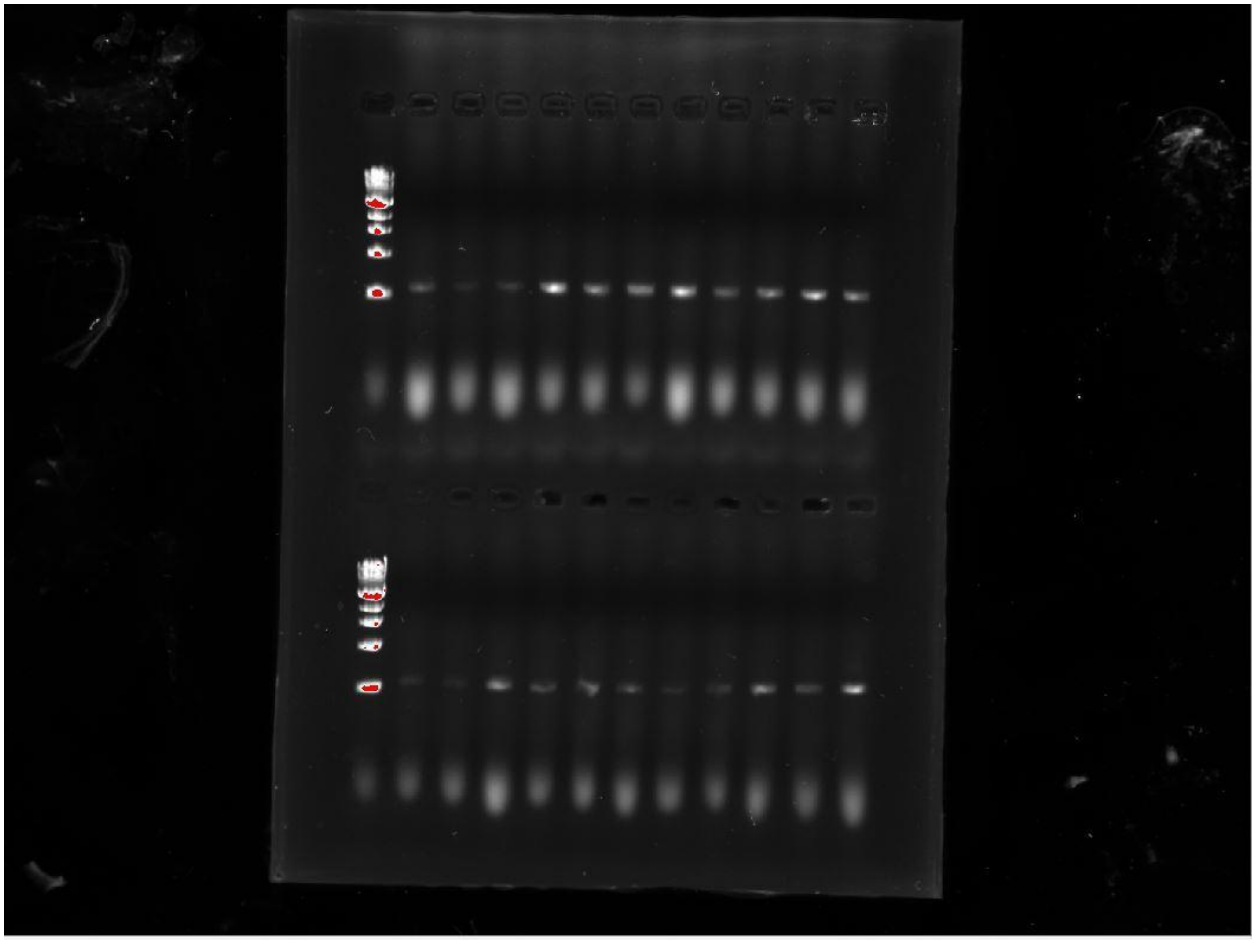


Figure 10. Sample Gel Product featuring the analysis of 22 DNA samples.

D. DNA Sequencing

Once 30 Exosap products were available for each bottle that was selected for testing, all of the Exosap products were transferred to multiple 96 sample PCR plates, and sent to the University of Chicago for DNA sequencing. Along with the Exosap samples, a 1.5 mL Eppendorf tube of NonA Forward Primer consisting of 2 uL per sample was prepared and sent as well.

E. Fly Identification

Once the samples had been sequenced, the data was analyzed utilizing the software program Geneious Prime. The NonA primer consists of a ~500bp fragment, with a four base pair segment around the 450bp mark which serves a purpose for fly identification. This sequence is conserved across the WN species with a segment containing AGCG, while the EA species is identified via a TCAA sequence. Hybrids were identified through their lack of a distinction between the two sequences and were most easily recognizable when relatively distinct base pairings were observed around the four base pair site, with the actual site retaining muddled base pair sequences.

Each fly that was sequenced was identified when possible, and the data was stored in an Excel spreadsheet via a distinct serial number given to each fly, designating its bottle of origin and individuality within that bottle.

Results

The Pure Species Control trial was pursued to establish whether these two pure species, WN and EA, who are the respective parental species of the hybrid populations used in other trials within this study, would outcross from within their own species with each other. Furthermore, any significant genotypic frequency deviations from the aforementioned fertilization models would be indicative of inherent competitive advantages or disadvantages between the two species, across the entire *Drosophila* life cycle. Upon initial DNA sequencing of the four replicate trials, no hybrid F1 offspring were identified. In fact, from the total 100 flies sequenced from these replicates, the exact genotypic frequency of each pure species was determined to be 0.5 **(Table 13)**.

While the individual replicates varied slightly in their respective genotypic frequencies, it is clear that these species are strongly sexually isolated and that no outcrossing between the species occurs. A Oneway ANOVA analysis of the four replicates was conducted to determine whether there was any significant difference between the genotypic frequencies of the two species, to which there was none **(Figure 11)**. Of course, there were significant differences between the genotypic frequencies of the pure species and hybrid populations as not a single hybrid was recorded among the four replicates (p-value < 0.05). Thus, it is clear that the pure species adhere closely to the Self-Fertilization Model by which no out-crossings occur, while the minimal deviations among replicate frequencies can most likely be attributed to random sampling. Furthermore, the lack of out-crossings in the pure species trial make it unlikely that any

out-crossings between these pure species occurred within the experimental trials (See **Figures 11 and 12** for Oneway ANOVA analysis and distribution model tests).

The Experimental Trial was tested in order to determine hybrid viability and mating success while inside of a competitive environment with the respective pure species. Each Experimental Replicate was thus composed of three separate fly species/populations inhabiting the same experimental bottle. Thus, the initial genotypic frequency of each fly type was assigned to be 0.33 as they were all in equal proportion. These experimental replicates were run in order to determine whether the hybrid population suffers in any manner in comparison to their parental pure species counterparts. Any outcrossings between the hybrid populations and either pure species could also be discerned by analyzing the F1 genotypic frequencies of the sampled offspring. Thus the F1 offspring were sequenced and organized by fly type and associated replicate, to test the fly type genotypic frequencies against the aforementioned Fertilization models. A Oneway ANOVA analysis (fly frequency by fly type) as well as three genotypic frequency distributions (and associated fertilization model t-tests) amongst the three separate fly types were conducted (See **Figures 13 and 14**).

From the Oneway ANOVA analysis it was found that there was a significant difference between the mean genotypic frequencies of the WN mix populations and the hybrid populations (p-value < 0.0227). With a mean genotypic frequency of ~0.2, the hybrids do not adhere to the Partial Self-Fertilization model which hypothesized a mean genotypic frequency of ~0.33 (**Table 1)**. To determine this significance, the Partial Self-Fertilization model t-test associated with the hybrid genotypic frequency distribution, found that the hybrid frequency of ~0.2 was significantly

different from the hypothesized ~0.33 (**Figure 14)**. This indicates that either the hybrid population was not very successful in outcrossing with the pure species or that the hybrids suffer in terms of viability as compared to the two pure species, or a combination of the two. The data indicates that the hybrid population within the experimental replicates adheres more closely to the Self-Fertilization model's hypothesized 0.165 value (to which it was not significantly different). The previously described Pure Species Control Replicates also elucidate the point that there was most likely no regeneration of hybrids through pure species outcrosses which is also evident by the low genotypic frequency of the hybrids.

In order to further elucidate any deviations from the aforementioned fertilization models, alternate trials were conducted in which a pure species was placed in equivalent proportion to one of the two hybrid subdivisions (EA derived hybrids, or WN derived hybrids). One replicate of each alternate combination was enacted: 1 EA mix x WNDH, 1 EA mix x EADH, 1 WN mix x WNDH, and 1 WN mix x EADH. By examining the genotypic frequencies of the F1 offspring of each of these alternate trials, competitive relationships and mating relationships or mating preferences between the sampled populations/species, as well as the subsequent deviations from the fertilization models could be noted. Thus each alternate trial bottle contained 30 pure species flies, and 30 hybrid subdivision flies, with each further subdivided equally by the representative sex (15 males, 15 females each).

Due to the subsequent sequencing of only one replicate of each alternate trial combination, statistical analyses would not be plausible in describing any significant trends or deviations from models which could potentially (anecdotally) appear within the data. Because of the possibility

of sampling error, or rather sampling bias (random selection of more flies of a certain type than truly representative of the total F1 population), it was predetermined that only general relationships and trends would be established from the forthcoming presented alternate trial data **(see Table 8** for data, and **Table 9** for compiled hypothesized model frequencies**).**

	EA mix F1 Genotypic Frequency	**Hybrid F1 Genotypic Frequency**	**WN mix F1 Genotypic Frequency**	Male Courtship Song Similarity between Species
WN mix x WNDH	**0**	**0.4**	**0.6**	Similar
WN mix x EADH	**0.392857143**	**0.25**	**0.357143**	Dissimilar
EA mix x WNDH	**0.357142857**	**0.392857**	**0.25**	Dissimilar
EA mix x EADH	**0.6**	**0.4**	**0**	Similar

Table 8. Raw averages from each alternate trial. The above table displays the raw averages of each fly type across its given trial (1 replicate each). Because of the X chromosome-linked male courtship song, the identity of the mothers of the hybrids are important, as their offspring will respond to or express their mother's species' respective male courtship song. Thus the rightmost column displays whether there is a similarity in male courtship song between the two assayed species/populations (i.e. WN mix x Hybrids born to West Northern mothers).

	EA mix F1 Genotypic Frequency	**Hybrid F1 Genotypic Frequency**	**WN mix F1 Genotypic Frequency**	Male Courtship Song Similarity between Species
WN mix x WNDH **(SF)**	**0.125**	**0.25**	**0.625**	**Similar**
WN mix x WNDH **(PSF)**	**0.09375**	**0.3125**	**0.594**	**Similar**
WN mix x EADH **(SF)**	**0.125**	**0.25**	**0.625**	**Dissimilar**
WN mix x EADH **(PSF)**	**0.09375**	**0.3125**	**0.594**	**Dissimilar**
EA mix x WNDH **(SF)**	**0.625**	**0.25**	**0.125**	**Dissimilar**
EA mix x WNDH **(PSF)**	**0.594**	**0.3125**	**0.09375**	**Dissimilar**
EA mix x EADH **(SF)**	**0.625**	**0.25**	**0.125**	**Similar**
EA mix x EADH **(PSF)**	**0.594**	**0.3125**	**0.09375**	**Similar**

Table 9. Compiled Hypothesized Genotypic Frequencies for each Fly type for each Alternate Replicate. SF - Self Fertilization Model, PSF - Partial Self Fertilization Model.
The alternate trials were specifically established to allow hybrids born to a specific pure species mother to be able to individually compete with and potentially mate with one of the two pure species. As male courtship song has been found to be both X-linked and autosomal linked, hybrids born to the mother of a specific pure species will go on to express and select upon their mother's pure species male courtship song (males and females, respectively) (Yukilevich et al. 2016). Thus, as visible in the rightmost columns of Tables 4 and 5, the alternate trials in which the male courtship song is common between the two tested populations is noted.

The two trials which shared a common expression of the male courtship song, EA mix x EADH and WN mix x WNDH, expressed a reciprocal relationship in terms of their genotypic frequencies. Both trials produced an F1 hybrid genotypic frequency of 0.4, and the respective pure species tested within that trial held a genotypic frequency of 0.6, with the absence of any opposing, non-directly tested pure species. On the other hand, the alternate trials sharing dissimilar expressions of the male courtship song, WN mix x EADH and EA mix x WNDH, displayed much greater genotypic frequencies of the non-directly tested pure species.

From these results, it can be hypothesized that the similar song alternate trials appear to adhere more closely with the Partial Self-Fertilization model. This is because the hybrid population self-fertilization model postulates that when the hybrid populations self-fertilize, the non-directly tested pure species (EA in the WN mix x WNDH, and WN in the EA mix x EADH) can only be regenerated through hybrid population self-fertilization. Because of the absence of non-directly tested pure species in the F1 generation , the hybrid populations must have been outcrossing with their respective pure species populations at a significantly high level. Thus the genetic linkage of the male courtship song with the pure species identity of the mothers which birthed those specifically tested hybrid subdivisions, can grant those hybrids the ability to successfully outcross with their mother's pure species as the associated male courtship song is preserved.

As previously mentioned however, the two alternate trials with dissimilar expression of male courtship song (WN mix x EADH and EA mix x WNDH) showed, relatively speaking, large F1 genotypic frequencies for the non-directly tested pure species (WN for EA mix x

WNDH, and EA for WN mix x EADH). Following the logic of a genetically linked male courtship song dependent on the hybrids' mother's associated pure species, the hybrid flies would have very low levels of mating success with their individual trial's tested pure species. This can be concluded from the results of the prior pure species control trial, in which no outcrossing occured between the two pure species across four replicates. Thus the hybrid flies of the dissimilar expression of male courtship song alternate trials could be found to adhere more closely to the Self-Fertilization model. As previously stated, the only way to regenerate a non-directly tested pure species within these alternate trials is through hybrid-hybrid matings. The, relatively speaking, large genotypic frequencies of the non-directly tested pure species seem to indicate that high levels of hybrid inbreeding occurred within these alternate trials. However, across the four alternate combination trials, the genotypic frequencies of the hybrids are relatively high, when compared to the idealized genotypic frequencies of both the Self-fertilization and Partial Self-fertilization models. Furthermore, they are not representative of the Experimental Trial's hybrid genotypic frequency (~0.20), indicating that the hybrids may be more successful in competition, or mating success when just placed in a micro-environment with one other pure species.

Thus, there appears to be an apparent contradiction between the genotypic frequency of the hybrid population in the Experimental Trial, as opposed to the hybrid's relatively larger genotypic frequencies among the alternate trials. This would seem to indicate that when paired with both pure species in an experimental replicate, the hybrid population suffers in relation to the competitive pure species populations. On the other hand, the alternate trials present hybrid's as much more vigorous, by being able to represent a larger portion of the F1 genotypic range.

Similarly to the Alternate combination Trial, the Fecundity Trial may lack in replicate sample size, but general trends appear to be apparent within the data. The Fecundity Trial involved the placement of one sole fly type within a bottled micro-environment, only subdivided equally by sex. The goal of these replicate trials was to determine whether there were any trends between the variety of offspring produced by each individual fly population. In order to even minutely examine these relationships, hybrids were analyzed by method of one-way analysis and respective t-tests by being paired into groups based on the specific male courtship expressed by both members of the pair. Thus **Figure 16**, represents the one-way analysis between the total fecundity (male and female offspring) of the replicate pair which both express the EA male courtship song. **Figure 17**, on the other hand represents the one-way analysis between the total fecundity (male and female offspring) of the replicate pair which both express the WN male courtship song. The data indicates that the hybrid populations produced more offspring on average than the pure species did. While the respective t-tests did not depict any significant differences within the two groups of data (p-value > 0.05), the mean offspring produced by the pure species, 137.5 and 141, and the mean offspring produced by the hybrid subdivisions, 192 and 192.5 are strikingly similar towards each other. This could be an indicator of hybrid vigor in terms of fecundity, which could account for the increased genotypic frequencies of non-directly tested pure species in the two dissimilar male courtship song expression alternate trials (WN mix x EADH, EA mix x WNDH). As the hybrid populations within those alternate trials could have possibly been forced to inbreed, the apparent vigor in fecundity could be responsible for those heightened aforementioned genotypic frequencies of the non-directly tested pure species.

Pure Species Control Results Figures

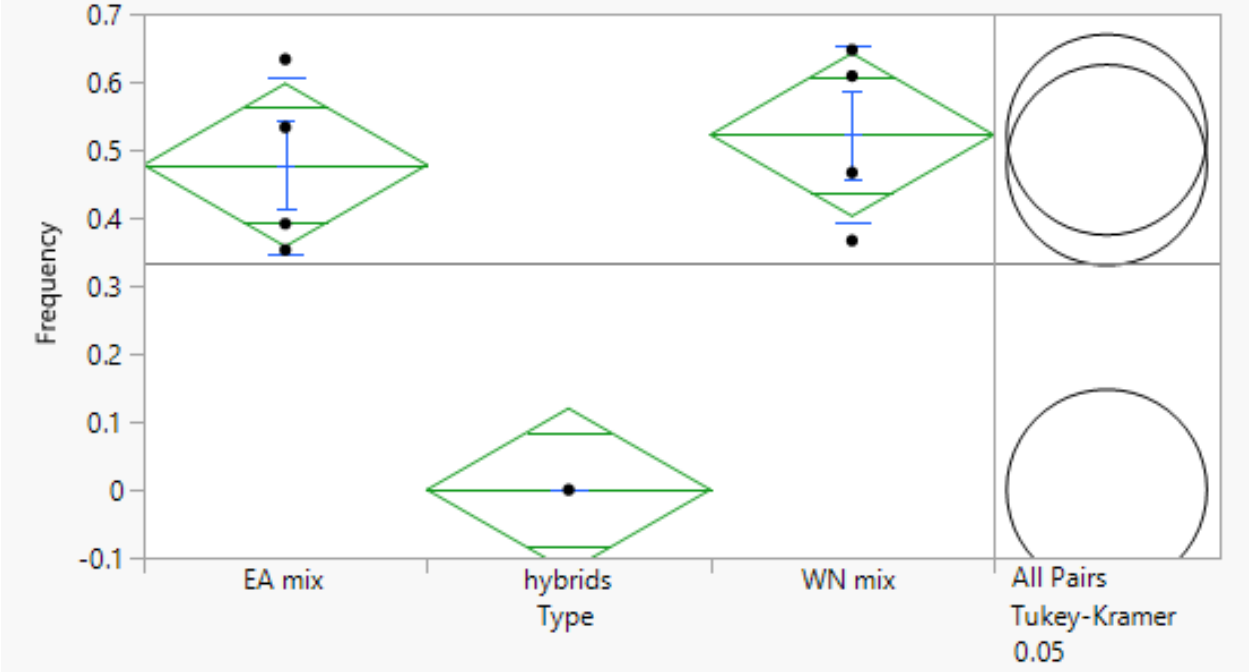


Figure 11. Oneway ANOVA of Pure Species Control Replicates, Genotypic Frequency by Fly Type. This above figure represents the oneway analysis of the genotypic frequencies of each fly type from the four pure species control replicates. No hybrids were found in neither of the four pure species replicates, thus the data expresses significant differences between the hybrids and either of the pure species
(p-Values: WN mix - <0.0002, EA mix - <0.0003).

Fly Type	# of Replicates (samples)	Mean	Standard Deviation
EA mix	**4**	**0.4777**	**0.1295**
Hybrid	**4**	**0**	**0**

WN mix	**4**	**0.5222**	**0.1295**

Table 10. Associated Means and Standard Deviations of Figure 11.

EA mix Distribution **WN mix Distribution**

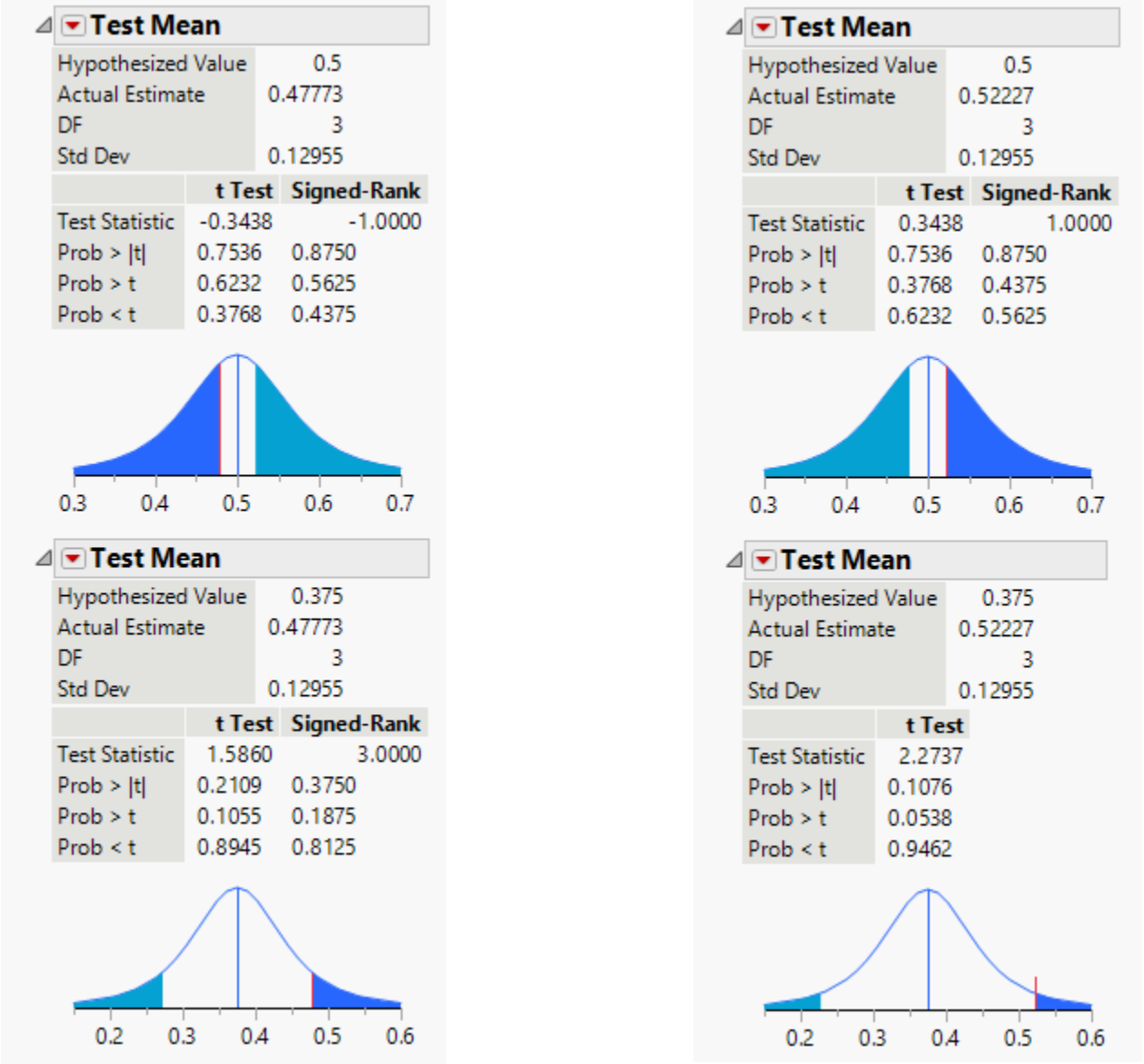


Figure 12a, 12b. Mean T-tests across Pure Species Control Replicates. The T-tests located at the top of each figure represent a test against the proposed **Self-Fertilization Hypothesis** with the respective **Hypothesized Value** representing each fly type's calculated prospective genotypic frequency. The **Actual Estimate** value represents the average genotypic frequency of the given

fly type across the experimental replicates. The data showed no significant differences from hypothesized and actual values for the **Self-Fertilization Model**. The T-tests located at the bottom of each figure represent a test against the proposed **Partial Self-Fertilization Hypothesis**, and the respective hypothesized genotypic frequency values. The Hybrid Mean T-tests are omitted as no hybrid flies were collected within any of the four Pure Species Control Replicates. However, no significant differences were found between any of the EA, hybrid, or WN averages and their respective hypothesized model values.

Experimental Results Figures

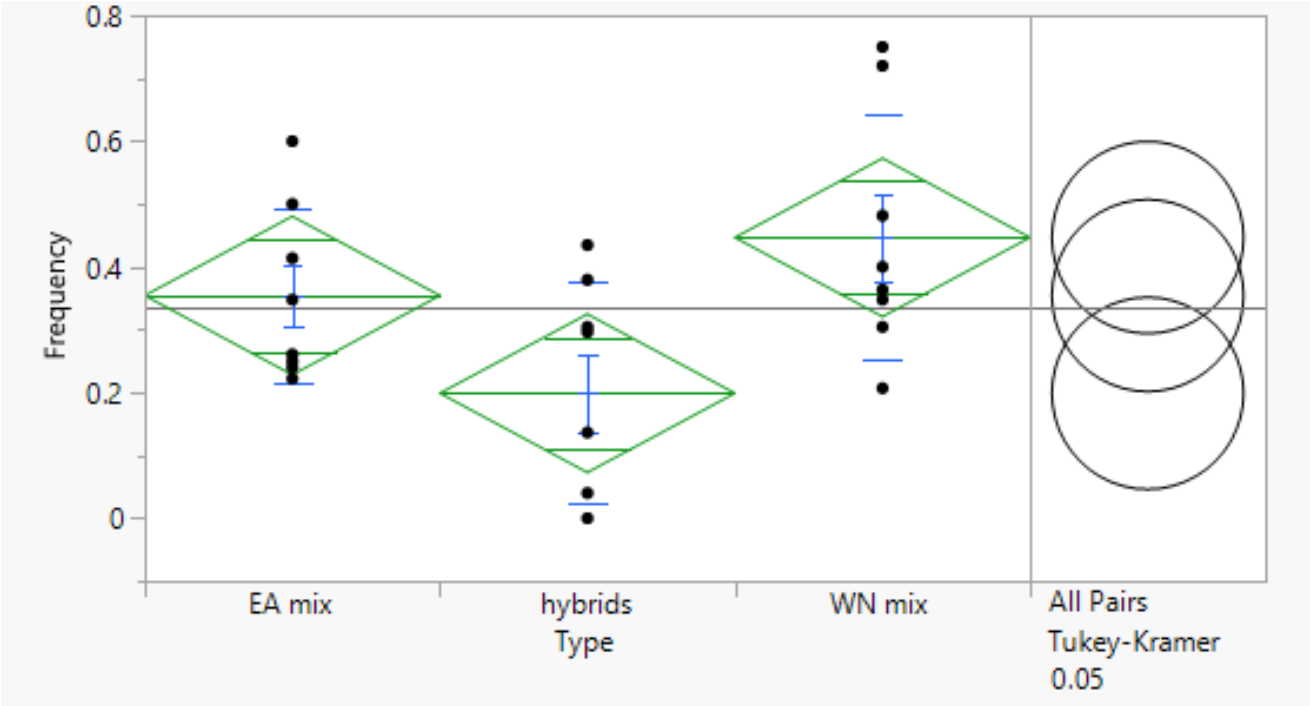


Figure 13. Oneway ANOVA of Experimental Replicates, Genotypic Frequency by Fly Type. This above figure represents the oneway analysis of the genotypic frequencies of each fly type from the eight experimental replicates.

Fly Type	# of Replicates (samples)	Mean	Standard Deviation
EA mix	**8**	**0.3543**	**0.1390**
Hybrid	**8**	**0.1988**	**0.1760**
WN mix	**8**	**0.4467**	**0.1943**

Table 11. Associated Means and Standard Deviations of Figure 6.

EA mix Distribution **Hybrid Distribution** **WN mix Distribution**

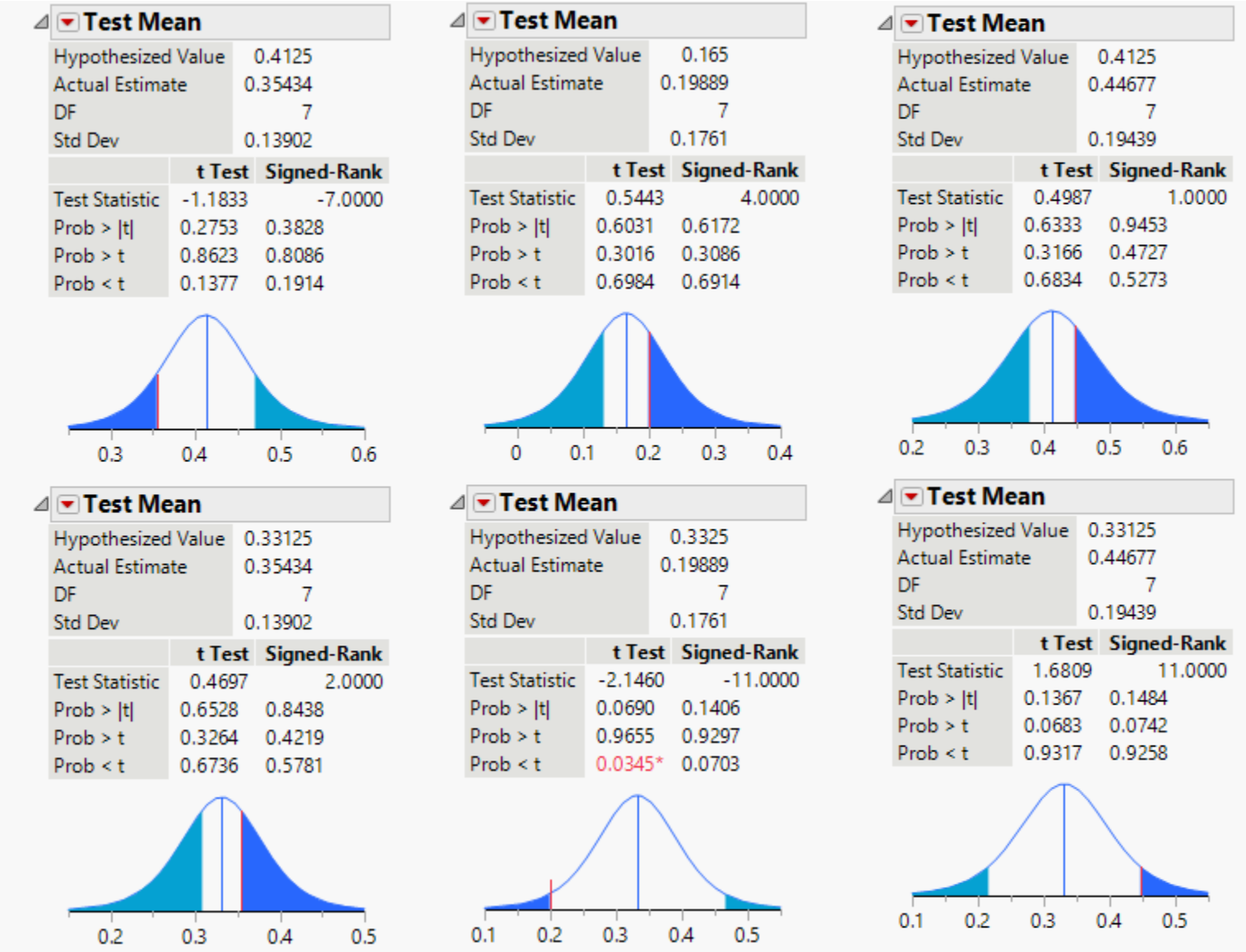
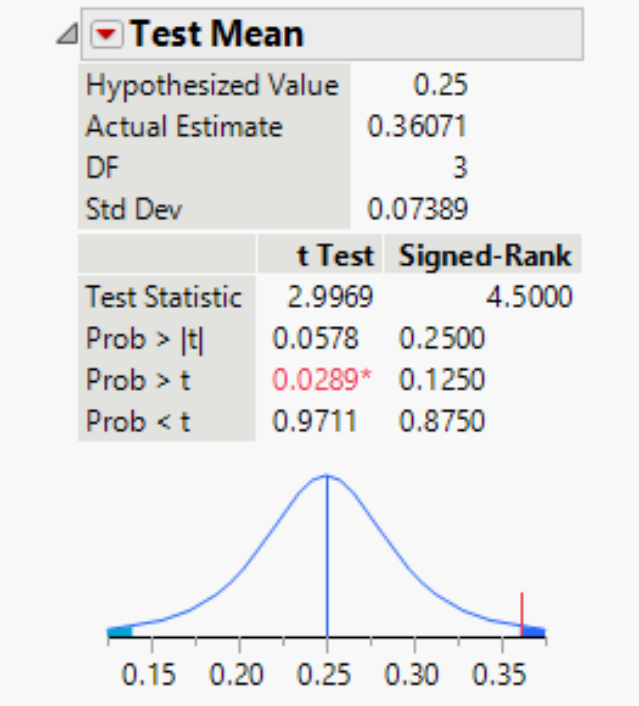


Figure 14. Mean T-tests across Experimental Replicate by Fly Type. The above figure describes the Mean T-tests across the distributions of the experimental replicates of the EA mix, Hybrid, and WN mix fly types. The T-tests located at the top of each figure represent a test against the proposed **Self-Fertilization Hypothesis** with the respective **Hypothesized Value** representing each fly type's calculated prospective genotypic frequency. The **Actual Estimate** value represents the average genotypic frequency of the given fly type across the experimental replicates. The data showed no significant differences from hypothesized and actual values for

the **Self-Fertilization Model**. The T-tests located at the bottom of each figure represent a test against the proposed **Partial Self-Fertilization Hypothesis**, and the respective hypothesized genotypic frequency values. The Hybrid distribution T-test for the Partial Self-Fertilization Model, is the only distribution to show a significant difference in a one way T-test (**p-value 0.0345**)

Alternate Trial Results Figures

For simplification, WNDH and EADH stand for West Northern derived hybrids, and Eastern A derived hybrids respectively, in order to indicate the identity of the mother to which the given hybrids were born.



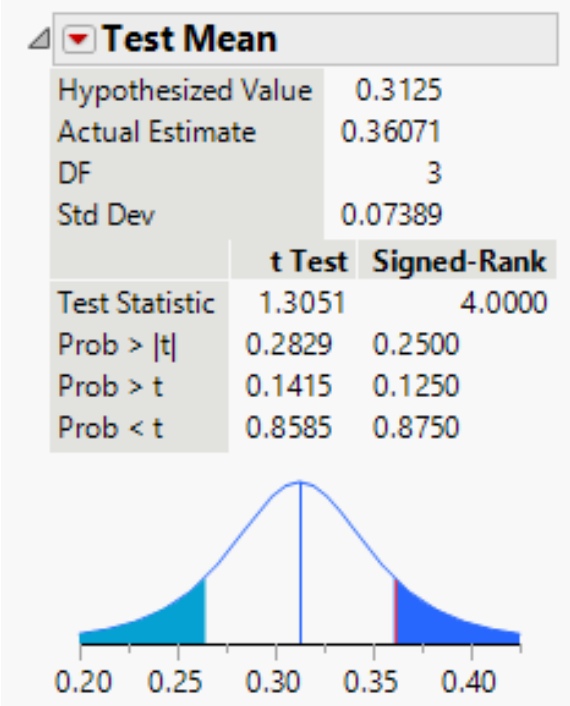
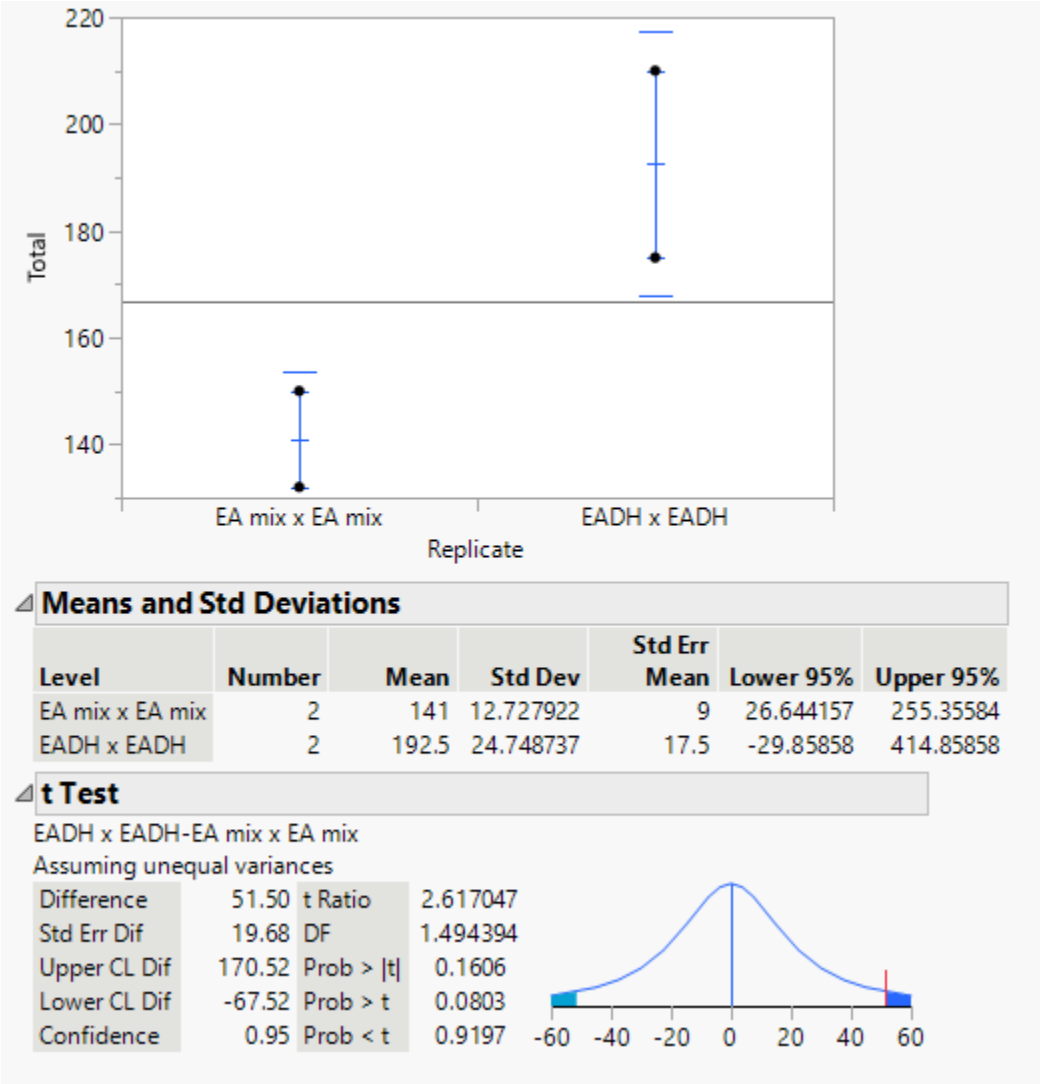


Figure 15a, 15b. T-Test of alternate replicates hybrid distribution. The above figures depict the results of two separate t-tests of the alternate replicates' hybrid genotypic frequency distribution. Figure 15a on the left depicts a significant difference between the estimated hybrid genotypic frequency and the hypothesized value calculated from the Self-Fertilization model **(p-value 0.0289)**. 11b on the right depicts no significant differences between the estimated hybrid genotypic frequency and the hypothesized value calculated from the Partial Self-Fertilization model.

Fecundity Results



Means and Std Deviations

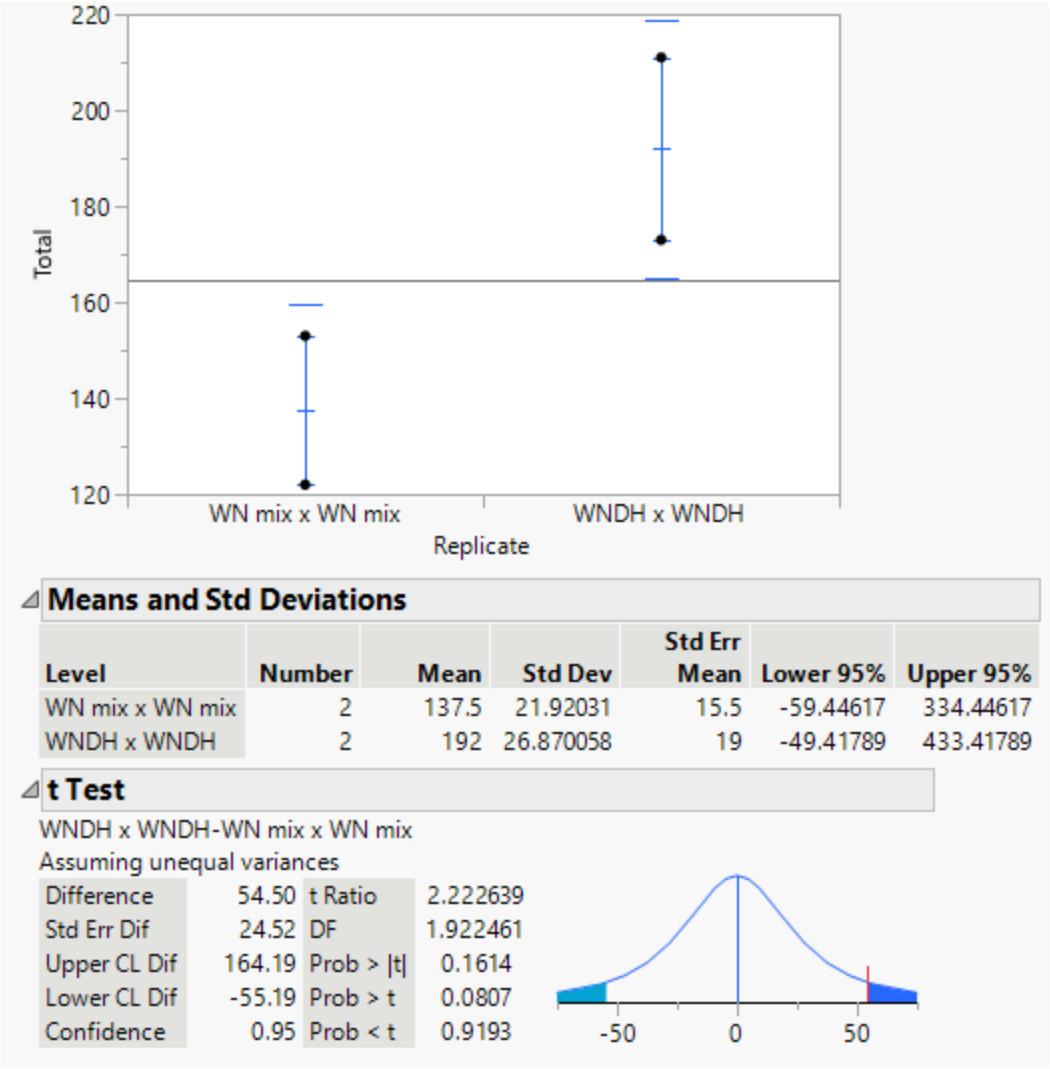
Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
EA mix x EA mix	2	141	12.727922	9	26.644157	255.35584
EADH x EADH	2	192.5	24.748737	17.5	-29.85858	414.85858

t Test

EADH x EADH-EA mix x EA mix
Assuming unequal variances

Difference	51.50	t Ratio	2.617047
Std Err Dif	19.68	DF	1.494394
Upper CL Dif	170.52	Prob > |t|	0.1606
Lower CL Dif	-67.52	Prob > t	0.0803
Confidence	0.95	Prob < t	0.9197

Figure 16. Oneway Analysis of Total Offspring Produced by Replicate, assorted via Similar Song Expression (EA male courtship song). The above figure depicts the analysis of total offspring produced by Fecundity replicates which express the same male courtship song.



Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
WN mix x WN mix	2	137.5	21.92031	15.5	-59.44617	334.44617
WNDH x WNDH	2	192	26.870058	19	-49.41789	433.41789

t Test

WNDH x WNDH-WN mix x WN mix
Assuming unequal variances

Difference	54.50	t Ratio	2.222639
Std Err Dif	24.52	DF	1.922461
Upper CL Dif	164.19	Prob > |t|	0.1614
Lower CL Dif	-55.19	Prob > t	0.0807
Confidence	0.95	Prob < t	0.9193

Figure 17. Oneway Analysis of Total Offspring Produced by Replicate, assorted via Similar Song Expression (WN male courtship song). The above figure depicts the analysis of total offspring produced by Fecundity replicates which express the same male courtship song.

Raw Numbers

- Final Analysis consisted of: **8 Experimental** Replicates, **4 Pure Species Control** Replicates, and **4 Alternate Hybrid** Replicates: **16 Total Replicates** analyzed.
- A total of **179 flies** were successfully sequenced and identified from across **8 separate experimental** replicates **(Table 12)**
- A total of **100 flies** were successfully sequenced and identified from across **4 separate pure species** replicates **(Table 13)**
- A total of **106 flies** were successfully sequenced and identified from across **4 separate alternate hybrid** replicates.
- Grand total: **385 flies**, successfully sequenced and identified.

Raw Experimental Averages (data amassed from across all 8 experimental replicates)

	Experimental EA	**Experimental Hybrid**	**Experimental WN**	**Total**
Flies	60	40	79	179
Frequency	0.335195531	0.223463687	0.441340782	1.0

Table 12. Raw Experimental Averages by Fly Type.

Raw Pure Species Averages (data amassed from across all 4 pure species replicates)

	EAmix	**WNmix**	**Total**
Flies	50	50	100
Frequency	0.5	0.5	1.0

Table 13. Raw Pure Species Averages by Fly Type.

Discussion

The Experimental phase involved eight separate replicate bottles, containing 60 flies each: 20 EA mix (10 male and female), 20 WN mix, and 20 Hybrid flies (10 born to WN mothers, 10 born to EA mothers, with 5 of each sex). These experimental replicates were arguably the most important of the entire study, providing the rarest competitive scenario, in which all three species/populations could be found within one micro-environment. According to the aforementioned results, only the hybrid mean (~.20) was significantly different when compared to the Partial Self-Fertilization model's hypothesized value (.3325). This indicates that the hybrids appear to suffer to some degree when placed in a random mating environment. This is because of the S and T values utilized within the model's calculation, by which S and T, self-fertilization and outcrossing respectively, were both given a 0.5 value. This is indicative of a fly having the same chance of being born from a "within population" mating as opposed to an outcross or "outside of a population" mating, thereby, effectively an environment consisting of random mating.

When looking at the Pure Species results, it is clear that sexual isolation breakdown between Eastern A and West Northern flies takes much longer than the allotted 5 days that they were given in the bottles before being cleared. No hybrids were collected and identified from the four separate pure species replicate trials and thus it is plausible to state that the two species, when alone without hybrids, adhere closer towards the Self-Fertilization model and do not outcross with each other.

Because of the significant difference of the Experimental Trials' hybrid's mean from the hypothesized Partial Self-Fertilization model's mean, it would seem that the Experimental Trial flies also adhere more towards the Self-Fertilization model (p-value <0.05). However, the hybrid mean was determined to be ~0.2, only minutely higher than the Self-Fertilization model's hypothesized 0.165 value. The association of the male courtship song with the X-chromosome could have prompted outcross matings to occur between a subdivision of hybrid flies and the species with which their mother is associated with. Such outcrossings would be indicative of the ~0.2 F1 hybrid genotype, as the hybrid flies would not only be mating within their own hybrid population. The dilemma of the hybrid flies lies in the fact that they lose half of their heterozygosity in the next generation upon in-breeding with other hybrids. Thus, in the experimental trials, the ideal scenario for a hybrid is to mate with either of the pure species in order to prevent a loss of heterozygosity and to ensure the hybrid offspring within the next generation are maintained. Due to the complete absence of hybrid offspring in any of the pure species control replicates however, it is highly unlikely that outcrossings between EA and WN populations were responsible for the heightened hybrid genotype frequency. Furthermore, upon analyzing the alternate trials in which there was a significant difference in the one-way t-test between the average hybrid genotypic frequency and the hypothesized value of the Self-Fertilization model, it appears as though hybrids within the alternate trials were more fit than expected, and in fact out-crossed more than even the Partial Self-Fertilization model would indicate.

Returning to the concept of reinforcement in driving greater sexual isolation between two species due to a selection on maladapted hybrids, the aforementioned study, specifically the

Experimental Trial results, have seemed to indicate that the hybrid populations suffer in respect to the parental species, and the parental species do not outcross with each other to form more hybrids. Thus it would appear that the process of reinforcement is in effect throughout these Experimental Trials. These trials sought to emulate the sympatric zone inhabited by both parental species, while introducing a population of hybrids as well. The low genotypic frequency of the F1 hybrid populations, in association with no outcrossing between both parental species is indicative of strong sexual isolation of the two species. While regular fertility, and at times generalized trends of hyper-fecundity in the hybrid populations have been observed, the low genotypic frequencies of the hybrids follow the patterns of reinforcement that would be expected in the original divergence of these two species. These two species may have diverged from each other through selection on specific characteristics as well as a change in ecology over time, and whilst interacting through the sympatric zone, sexual isolation with maladapted hybrids which may have not been suited towards the specific environment may have allowed for reinforcement to take effect.

From the aforementioned data, results and generalized conclusions, hybrid populations are found to adhere to the Self-Fertilization model of inbreeding when placed in a competitive environment with their parental, pure species. While, potential hybrid vigor in fecundity, and apparent success in outcross mating and maintenance of F1 hybrid genotypic frequencies in alternate combination trials have both been observed throughout this study, replicate trials of the aforementioned methodology should be pursued to either bolster or diminish the conclusion that hybrids suffer in comparison to their pure species counterparts. Unfortunately, due to time constraints, not all randomly selected flies from all bottle replicates were sequenced. Thus, the

remaining flies, especially in the alternate combination replicates may serve to further elucidate any trends of either hybrid vigor or suffering when in competition with the pure species. However, this first depiction of a competitive environment between the three species seems to indicate that the hybrids, as hypothesized, suffer as compared to their pure, parental species. This was indicated by the lower proportions of hybrids in the F1 generations within the experimental trials. Subsequent generational trials within the aforementioned study would have most likely led to a further decline in hybrid genotypic frequencies across the experimental trials due to their adherence to the Self-Fertilization model.

Future studies could also focus on the unanswered questions related to *Drosophila* ecology, and how the variation of factors such as food substance, temperature, or humidity could affect genotypic frequencies among experimental trials.

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