A Multi-Faceted Analysis of Spinosad Resistance in the House Fly (*Musca domestica* L.)

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Abstract

House flies are important vectors of a number of animal and human pathogens, thus controlling them is of great importance. Commercial insecticide control of house flies is limited to a few chemicals, and the development of resistance to these chemicals is an ongoing concern. Therefore, finding new effective insecticides is critical.

Spinosad is a relatively new promising insecticide that was released in 2005 for house fly control. It is derived from the bacterium *Saccharopolyspora spinosa*. It is highly effective against pest species and is thought to have a unique mode of action. A resistant laboratory house fly strain was developed and studies indicate the resistance mechanism is unique, recessive, and located on autosome 1.

Although house fly resistance to spinosad has been developed in the laboratory, resistance in field populations has not been characterized. In this study I monitored spinosad resistance at several dairy, hog and poultry farms over the summers of 2004 and 2005. Results showed that there was a variation in baseline susceptibility between different field sites, but no development of resistance was observed. Due to limitations of insecticide bioassays, developing a more sensitive resistance detection method and identifying the gene responsible for resistance will be important for future monitoring programs.

As part of an important step in determining the gene for resistance, I linked a number of nicotinic acetylcholine receptor (nAChR) subunits to a particular house fly autosome. Spinosad toxicity is due to interactions with nAChRs and is associated with autosome 1. Of the four genes analyzed, two (*Mdα5* and *Mdα6)* were associated with

autosome 1, one (*Mdα2)* was associated with autosome 2, and one gene association (*Mdβ3*) was not determined.

Lastly, I assessed the fitness effects of spinosad resistance on mating competition. I found that a laboratory susceptible strain had a mating advantage over a laboratory spinosad resistant strain.

Chapter 1: Introduction

1.1 Importance of the House Fly and its Control

House flies are important vectors of diseases. They have been found to carry and transmit the deadly bacteria *Escherichia coli* O157:H7 (Iwasa et al. 1999, Sasaki et al. 2000), and are likely vectors for over sixty five human and animal intestinal diseases (Greenberg 1965). A more recent study found that house flies act as mechanical vectors of *Vibrio cholerae*, the pathogen responsible for cholera (Fotedar 2001). Therefore, the control of house fly (*Musca domestica* L.) populations is of extreme importance.

The most common method for control of house flies is through the use of insecticides. Over the past seventy years, a variety of chemicals have been used to control house flies including chlorinated hydrocarbons, organophosphates, carbamates and pyrethroids (Shono et al. 2004). Today, commercial house fly control is limited to a few organophosphates, one carbamate (methomyl), pyrethrins and two pyrethroids . Unfortunately, house fly populations can rapidly evolve resistance to insecticides, which limits our ability to control them.

Resistance in house flies has become a global problem (Pospischil et al. 1996, Keiding 1999, Cao et al. 2006). This problem is exemplified with the widespread resistance to organophosphates (OPs) and pyrethroids. OP resistance is prevalent and aptly summarized in Keiding's review (1999). A house fly survey in 1999 showed that resistance to permethrin in particular has increased since 1987 in New York dairies (Kaufman et al. 2001). In certain New York poultry facilities, resistance has severely decreased the efficacy of permethrin, cyfluthrin and pyrethrins (Scott et al. 2000).

1.2 General Background on Insecticide Resistance in House Flies

Resistance to insecticides has been documented in over four hundred arthropod species (Georghiou and Mellon 1983). This phenomenon arises from three causes: target site insensitivity, metabolic detoxification and/or decreased penetration (Oppenoorth 1982).

Resistance to organophosphate insecticides, for example, is often the result of target site insensitivity. Organophosphates inhibit the enzyme acetylcholinesterase (AChE), which hydrolyzes the neurotransmitter acetylcholine (Eldefrawi 1985). House fly resistance to these insecticides is a result of insensitivity of AChEs to the compounds through mutations in the gene coding for the enzyme (Tripathi and O'Brien 1973, Kozaki et al. 2001, Walsh et al. 2001). Insecticide resistance through increased metabolic detoxification, primarily from P450 monooxygenase or esterase activity, is one of the most important mechanisms of insecticide resistance (Oppenoorth 1985). Pyrethroid resistance in both field and lab populations of the house fly is primarily due to increased levels of a P450 (Scott and Georghiou 1986) specifically, CYP6D1 (Wheelock and Scott 1990, 1992, Zhang and Scott 1996, Kasai and Scott 2000). Lastly, decreased penetration has been found to be a resistance mechanism in pyrethroid and organophosphorous resistant laboratory strains (Plapp and Hoyer 1968, Scott and Georghiou 1986, Shono et al. 2002) .

1.3 How We Control Resistance

As outlined by Georghiou (1983), resistance management takes on three main forms: moderation, saturation and multiple attack. With the moderation tactic, one sprays as little as possible so as not to push the population to resistance. If the selection pressure is kept at a minimum, susceptible alleles will remain in the population and thus dilute any possible resistance alleles. A saturation tactic is used if strongly controlling the pest is absolutely essential, such as in ornamental crops. Basically a high dose is used so as to kill individuals with both susceptible and resistance alleles. In multiple attack, different insecticides with different mechanisms of action are rotated or mixed. Use of insecticides in rotation is often the only practical resistance strategy. However, the lack of new insecticides, especially those acting on novel target sites, is a major limitation to resistance management.

1.4 Spinosad Insecticide

Spinosad is a relatively new insecticide (Saunders and Bret 1997), introduced by DowAgroSciences in 1997 for control of lepidopteran pests (Salgado et al. 1998). It is a fermentation product of the bacterium *Saccharopolyspora spinosa*. Spinosyns A and D are the two most active components, spinosyn A being the dominant metabolite (Kirst et al. 1991). Spinosad is unique in its class due to its high level of activity, which is comparable to synthetic insecticides (Bret et al. 1997b). Much like other natural insecticides, spinosad easily degrades under sunlight. Half lives for spinosyns A and D are 1.6 to 16 days and less than 7 days, respectively, depending on light exposure (Saunders and Bret 1997, Thompson et al. 2002). Studies of spinosad interaction with

the soil suggested that there is no concern of leaching (Thompson et al. 2002). The chemical does not have any detrimental impacts to non-target predators, but may pose sub-lethal problems for parasitoids (Williams et al. 2003).

Spinosad is thought to have a unique mode of action. Exposure to spinosad produces involuntary muscle contractions and tremors followed by paralysis (Salgado 1998). This suggests that the chemical interacts with the nervous system, causing over excitement and death. *Manduca* spp. ganglia show greater sensitivity than *M. domestica* L. or American cockroach (*Periplaneta americana*), consistent with greatest efficacy against lepidoptera (Salgado et al. 1998). The toxicity of spinosad is due primarily to interactions with nicotinic acetylcholine receptors (nAChRs) with a secondary site of action being GABA receptors (Salgado 1997, Salgado and Sparks 2005). Spinosad resistant laboratory house flies were not cross-resistant to other insecticides, supporting a unique resistance mechanism. This resistance mechanism was linked to autosome 1 (Shono and Scott 2003).

Although house fly resistance to spinosad has been selected for in the laboratory, resistance in field strains has not been characterized. The gene responsible for this resistance, as well as any fitness costs associated with resistance, are similarly unknown. These are all areas that the following research report will explore.

Chapter 2: Research Goals

In this study, I examined questions regarding spinosad resistance in *M. domestica* L. Three general areas of interest were examined: 1) The monitoring of resistance in the field. 2) Identification of nicotinic acetylcholine receptor subunits that might play a role in spinosad resistance. 3) The fitness effects of spinosad resistance.

The driving question behind the first area of interest was: Does spinosad resistance develop quickly in the field? One preliminary indication that it might, arose when a field-collected strain was selected in the lab to >150 fold resistance after 10 generations (Shono and Scott 2003). Since 10 field generations could easily pass over the course of a summer, I hypothesized that we would see a similar rapid development of resistance in field populations that were controlled using spinosad at dairy farms over one summer.

Much evidence suggests that resistance is due to a modification in the target site. It is first of all known that the toxicity of spinosad is due primarily to interactions with nAChRs (Salgado 1997). Also, metabolic detoxification, which is a common cause of insecticide resistance, does not play a role in spinosad resistance in the house fly (Zhao et al. 2002, Shono and Scott 2003). The most recent evidence indicates that spinosad resistance can be induced in *Drosophila melanogaster* through deletion of *Dα6* nAChR subunit (Perry et al. 2007a). All these supporting details led me to the question; what is the modification in the house fly nicotinic acetylcholine receptor that causes resistance? This was an important question, as determining a molecular basis for resistance would be useful in monitoring resistance in field populations. Since the location of the resistance

was already linked to autosome 1 (Shono and Scott 2003), linking potential resistance genes to a particular autosome would be useful in further identifying good candidate resistance genes. Linkage analysis would be an important preliminary step in the process of determining which gene mutation results in resistance.

The last area of interest is the fitness effects of spinosad resistance. The key question I wanted to examine was, how does spinosad resistance impact life history traits such as mating? Previous mating competition studies, in the *Culex pipiens* mosquito for example, have shown that insecticide resistance comes at a cost (Berticat et al. 2002). I hypothesized that I would similarly observe susceptible males out-competing resistant males. Understanding the relationship between resistance and life history could be important in assessing the weaknesses of resistant field populations.

Chapter 3: Resistance Monitoring in the Field

Note: This chapter has been accepted for publication (Deacutis et al. 2007), and thus the work of other individuals must be credited. In 2004, the Florida house flies were collected by Christopher J. Geden, the New York house flies were collected by C. Reasor, and the North Carolina house flies were collected by Wes D. Watson. In 2005, Alec C. Gerry collected the house flies from California, and Donald A. Rutz collected the flies from New York. The feeding bioassays were conducted by Cheryl A. Leichter.

3.1 Introduction

House flies, *M. domestica* L. (Diptera: Muscidae), are major pests in and around dairy, poultry and hog facilities. Given that resistance to organophosphate and pyrethroid insecticides in house flies in the USA is widespread (Scott et al. 1989, Scott et al. 2000, Kaufman and Rutz 2001, Kaufman et al. 2001, Darbro and Mullens 2004) there is an urgent need for new insecticides that are effective against this pest.

Spinosad is a new and highly promising insecticide, derived from the soil actinomycete *S. spinosa*. In 2005, spinosad was made available for control of house flies in the USA. Spinosad acts at the nicotinic acetylcholine receptor and has efficacy against a wide range of insects, including house flies (Bret et al. 1997a, Scott 1998, Salgado and Sparks 2005). Recently, field collected house flies were selected for resistance (in the laboratory), which developed a strain of house fly (NYSPINR) that had high levels of resistance to spinosad (Shono and Scott 2003). This indicates that there is potential for the evolution of resistance following repeated use of spinosad in the field. Isolation of

the NYSPINR strain required only ten generations of selection, suggesting resistance might be able to evolve in as little a one season. However, there is no information about the baseline susceptibility of field populations of house flies to spinosad, and no method in place for resistance monitoring.

The goals of this study were to identify an effective bioassay method for detection of spinosad resistant house flies, to survey for baseline susceptibility to spinosad in field collected house flies (i.e. determine variability between populations) in 2004 and 2005, and to determine if we could detect any increase in the frequency of resistant individuals at three dairies in California and three dairies in New York following a season of spinosad use (2005).

3.2 Materials and Methods

3.2.1 House flies

Two reference (laboratory) strains of house flies were used for comparison of topical, residual and feeding bioassays (below). CS (Hamm et al. 2005) is a strain broadly susceptible to insecticides and NYSPINR is a spinosad-resistant strain (Shono and Scott 2003). To produce flies that were heterozygous for spinosad resistance (for bioassays) we crossed NYSPINR females and susceptible aabys males, a mutant strain containing morphological markers (Hamm et al. 2005), and en masse.

In 2004 (prior to spinosad use), six different strains of house flies (*M. domestica* L.) were collected at various dairy (Alachua County, Florida; Schuyler and Tompkins Counties, New York), poultry (Sullivan and Wayne Counties, New York) and hog (Wake County, North Carolina) facilities in the Eastern United States. The levels of resistance

to permethrin, cyfluthrin, pyrethrins, dimethoate, tetrachlorvinphos and methomyl in house flies from the Schuyler County dairy and Wayne County poultry facility have been previously reported (Scott et al. 2000, Kaufman et al. 2001).

In 2005, house flies were collected from four dairies (P, M, H and C) in Tompkins County New York and from four dairies in San Diego (DV), San Bernardino (AM and BJ) and Riverside (BS) Counties, California. These facilities were chosen because we they were willing to participate in this study, they were within collecting distance, and because they represented two geographically distant regions (California and New York). Two collections were made at each dairy. The first collection was made before spinosad was used ("pre-season"). A second collection was made at the end of the season ("post-season"), but while flies were still abundant. In New York, the dairies applied up to 6 applications of spinosad, except for the H dairy that served as our no spinosad control. In California, dairies applied spinosad 4-5 times, except for the DV dairy which served as our no spinosad control.

House fly larvae were reared on medium containing 2.3 liters of water, 0.5 kg calf manna (Manna Pro Corp, St. Louis, Missouri), 90 g bird and reptile little wood chips (Northeastern Products Corp, Warnersburg, New York), 0.8 kg wheat bran (Agway; Ithaca, New York), and 50 g dried active baker's yeast (ICN Biomedicals, Costa Mesa, California). Adult flies were raised on a mixture of powdered milk and white granulated sugar (1:1 ratio by volume) as well as water, *ad libitum*.

3.2.2 Bioassays

Three bioassay methods were evaluated in this study: topical application to the thoracic notum in 0.5 µl of acetone (Shono and Scott 2003), residual exposure in glass

jars (Hamm et al. 2005) and feeding. For feeding assays, spinosad (spinosyns A and D (88.5% purity) from Dow AgroSciences, Indianapolis Indiana) was applied (0.25 ml in acetone solution) to individual cubes of sugar (Domino Dots, Tate and Lyle, London, United Kingdom). Treated cubes were allowed to dry for at least three hours. One cube of sugar was placed into a 180 ml Sweetheart waxed paper cup with 20 flies and a 2.5 cm dental wick soaked in water. Cups were covered with nylon tulle and secured with rubber bands. All bioassays were conducted with 3-5 d old female flies held at 25°C.

Mortality was assessed after 48 hr with flies that were ataxic being scored as dead. For determination of LD_{50} or LC_{50} values using the laboratory strains, a minimum of four doses (or concentrations), giving >0% and <100% mortality, were used for each replication and the entire bioassay was replicated a minimum of three times. Bioassay data were pooled and analyzed by standard probit analysis (Finney 1971), as adapted to personal computer use by Raymond (Raymond 1985) using Abbott's (Abbott 1925) correction for control mortality.

Field collected flies were tested within four generations of being collected. Field collected flies were bioassayed by topical application at the LD_{99}, 3 X LD_{99} and 10 X LD_{99} of the susceptible strain. Controls were treated with acetone. CS flies were periodically tested side-by-side with the field collected flies. Percent mortality was arc-sine transformed and pairwise differences were evaluated using Students t-test.

3.3 Results and Discussion

Spinosad was toxic to house flies by feeding, exposure to a residue, and topical application (Table 3.1). The NYSPINR strain was resistant to spinosad by all three methods (Table 3.1). These results are consistent with target site insensitivity being the mechanism of resistance (Scott 1990), as was previously suggested (Shono and Scott 2003). Considering the cost (supplies, etc.), efficiency (time per assay) and heterogeneity of response (i.e. slope) between the three bioassay methods, we chose to use topical application (low cost, time efficient and a low heterogeneity of response) at three diagnostic doses to monitor resistance in field populations of house flies.

To generate baseline data for the effectiveness of spinosad against field collected house flies, we determined the percent survival at three diagnostic concentrations (susceptible strain LD_{99}, 3 X LD_{99} and 10 X LD_{99}) by topical application. Susceptibility of the different house fly strains to spinosad varied between collection sites (Figure 3.1). At the susceptible strain LD_{99}, survival ranged from 1% (Alachua Co., Florida) to 61% (Wayne Co., New York), with all except one facility having <30% survival. At 3X LD_{99}, survival ranged from 0% (three sites) to 2% (Wayne Co., New York). There were no survivors from any strain at 10 X LD_{99}. The higher percent survival at the Wayne Co. New York site (at the LD_{99} and 3 X LD_{99}) suggests that there may be populations of house flies against which spinosad is less effective. Flies from the Florida dairy were highly susceptible, with few survivors at any dose. Although the highest percent survival was seen at two dairies, there was no correlation between type of facility and percent survival.

Evaluation of the New York dairies in 2005, prior to the use of spinosad, showed variability in response to spinosad similar to what was seen in 2004 with percent survival ranging from 16-21% at the susceptible strain LD_{99} (Figure 3.2). However, survival of flies from the four California dairies was lower, ranging from 0.5-3.0%. Following the use of spinosad for fly control during 2005, there was no indication that resistance was evolving (Figure 3.2). To the contrary, the survival following a season of spinosad use was significantly lower at two of the collection sites. It is unknown why the flies collected in California were more sensitive (in most cases) than flies collected in New York.

To evaluate the sensitivity of our resistance monitoring bioassay for detection of homozygous and heterozygous resistant individuals, we evaluated survival of NYSPINR and F_1 (NYSPINR females x aabys males) house flies. The NYSPINR strain had nearly 100% survival at the LD_{99} and 3 x LD_{99} doses, while the F_1 had 19% and 0% survival at these doses, respectively (Figure 3.1). Thus, the homozygous resistant house flies (NYSPINR) are readily detected, but the heterozygous resistant house flies (NYSPINR x aabys F_1) are indistinguishable from field collected house flies that have never been exposed to spinosad (Figure 3.1). The highly recessive nature of this resistance (combined with inherent variability in the bioassay with field collected flies) will make it very difficult to detect heterozygous resistant individuals (at least when they are present at low frequencies) in field populations.

Our results indicate that there is variation in susceptibility to spinosad in flies collected from different sites. While selection of field collected house flies produced a highly resistant strain of house fly following eight generations of selection (Shono and

Scott 2003), we did not detect a decrease in percent survival at the diagnostic dose at any site following one season of use. Spinosad works at a novel target site (Salgado and Sparks 2005) and resistance in the house fly is highly recessive (Shono and Scott 2003) which would be expected to slow the rate of evolution of resistance in field populations (Georghiou 1983). However, spinosad must be used judiciously and periodic monitoring of resistance should continue. Spinosad resistance is highly recessive and heterozygous individuals can not be readily detected (especially against the normal variation that exists in populations) using insecticide bioassays. Given this limitation, it will be important to identify the gene (and allele) responsible for spinosad resistance so that a more sensitive detection method can be developed.

Table 3.1. Comparison of spinosad toxicity to susceptible (CS) and resistant (NYSPINR) strains of house fly by three bioassay methods.

	CS		NYSPINR	
METHOD	**LC_{50} OR LD_{50} (CI)**	**SLOPE (SE)**	**LC_{50} OR LD_{50}**	**RR**
TOPICAL	**0.054[A] (0.049-0.058)**	**5.8 (0.9)**	**>10[B]**	**>150**
FEEDING	**2.85[C] (2.53-3.30)**	**3.0 (0.3)**	**>1000[D]**	**>300**
RESIDUE	**0.064[E] (0.038-0.108)**	**3.1 (1.6)**	**>60[F]**	**>900**

[a] LD_{50} in units of μg/fly at 48 hr (Shono and Scott 2003).

[b] Less than 50% mortality at 10 μg/fly.

[c] LC_{50} in units of μg/g at 48 hr.

[d] Less than 50% mortality at 1,000 μg/g.

[e] LC_{50} in units of μg/cm^2 at 48 hr.

[f] Less than 50% mortality at 60 μg/cm^2.

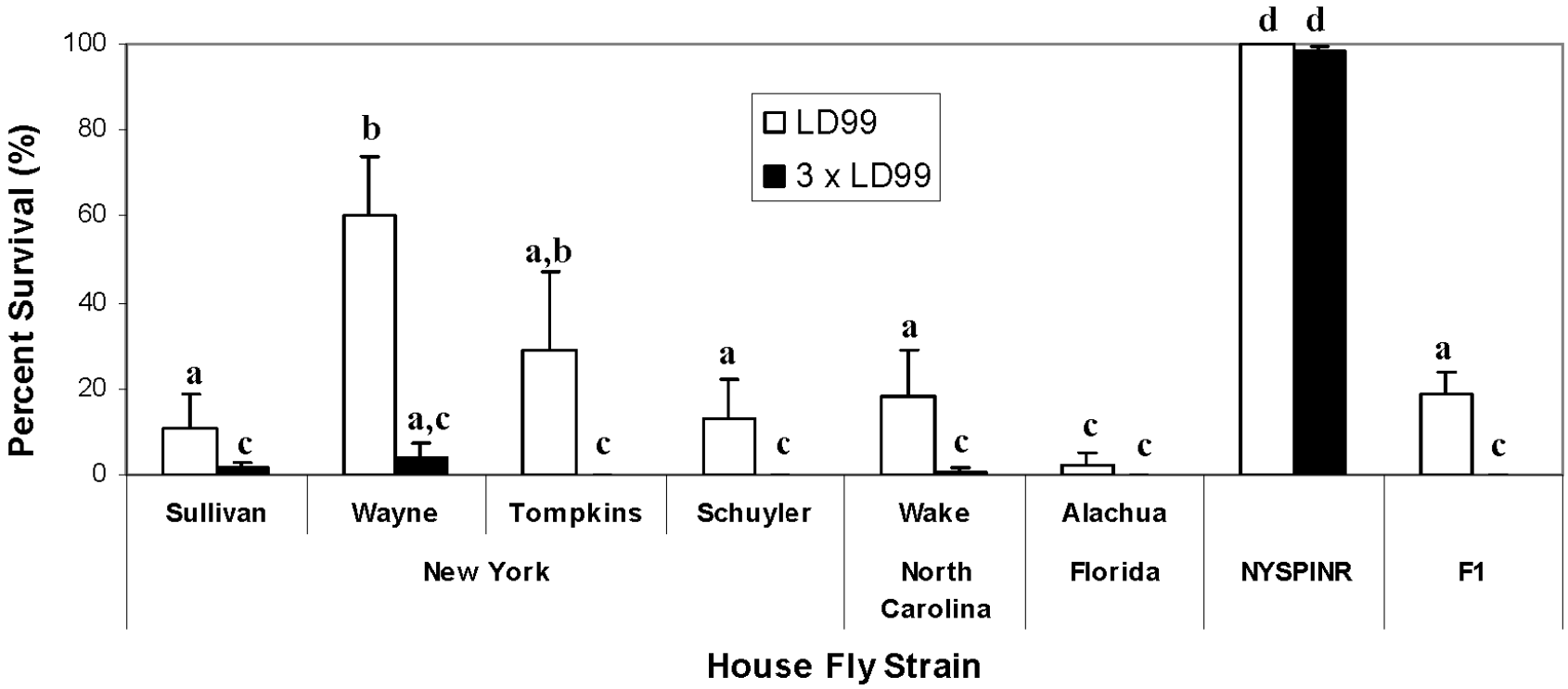

Figure 3.1. Mean percent survival of house flies collected in 2004 from six sites in the eastern United States at the susceptible strain LD_{99} and 3 x LD_{99}. All flies were from dairies, except for Wake (hog), Sullivan and Wayne (poultry) counties. The spinosad resistant NYSPINR and F_1 (NYSPINR x susceptible aabys strain) are shown for comparison. Bars represent the standard deviation from the mean. CS house flies treated at these doses had 0% survival.

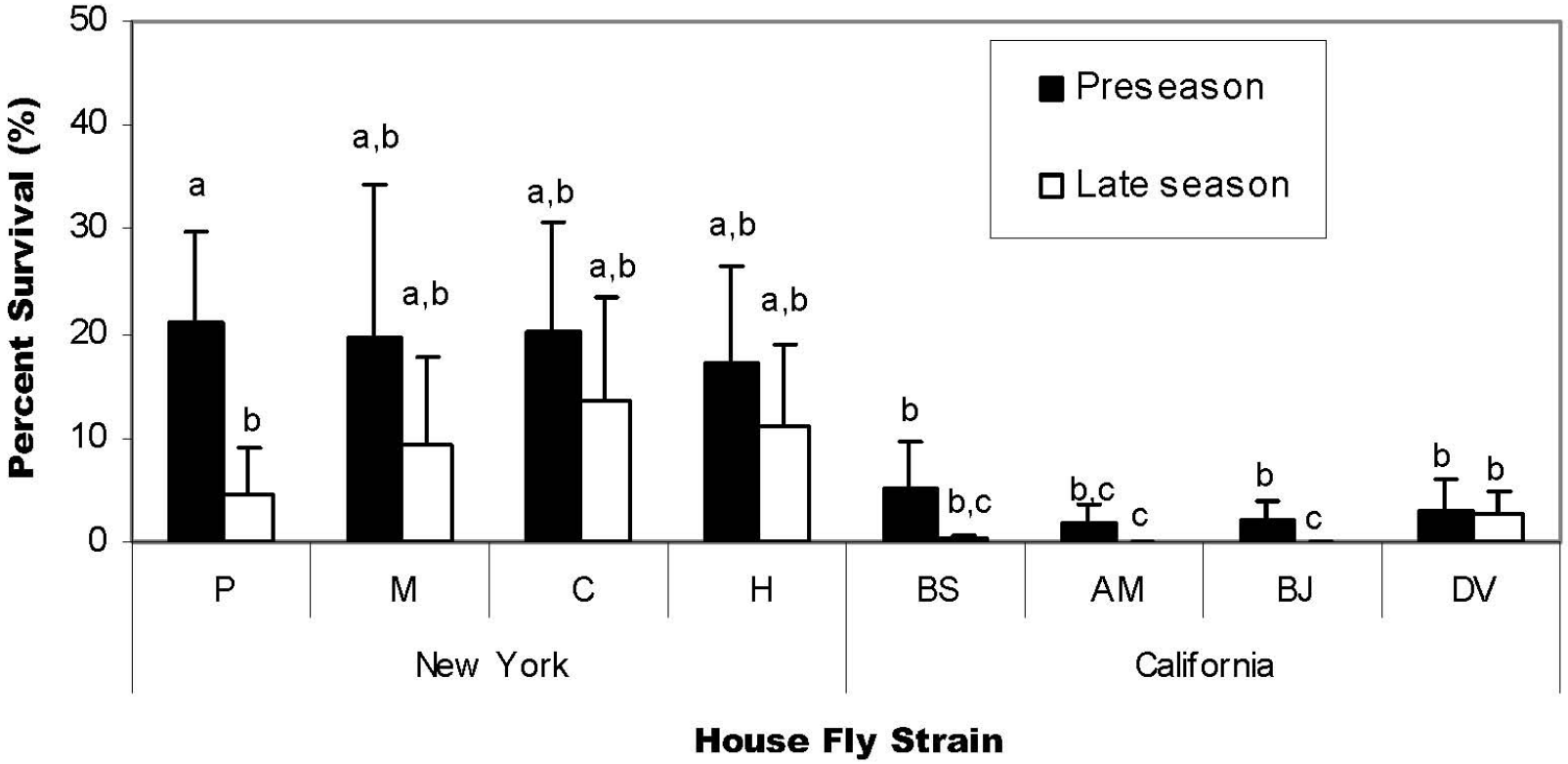

Figure 3.2. Mean percent survival of house flies collected in 2005 before (preseason) or after (lateseason) a season using spinosad for fly control, except for the H and DV dairies which served as negative controls (no spinosad was used at these dairies). Flies were tested at the susceptible strain LD_{99}. Bars represent the standard deviation from the mean.

Chapter 4: Linkage Analysis

4.1 Introduction

The nicotinic acetylcholine receptor (nAChR) is a ligand gated ion channel that plays an essential role in the fast excitatory neurotransmission at cholinergic synapses in the insect central nervous system (Lester et al. 2004), (Gundelfinger and Schulz 2000). The nAChRs are composed of five subunits, typically two α and three non-α subunits, but receptors composed of only α subunits are known (Couturier et al. 1990); (Marshall et al. 1990).

The toxicity of spinosad is due primarily to interactions with nAchRs (Watson 2001), and spinosad resistance is associated with autosome 1 (Shono and Scott 2003). Thus, associating the receptor subunit genes of *M. domestica* L. serves as an important step in determining the gene that confers resistance.

Several other linkage analyses have been completed using techniques similar to the ones used below. One study linked house fly acetylcholineesterase, which is competitively inhibited by organophosphorate and carbamate insecticides, to autosome 2 (Kozaki et al. 2002). Another study associated a phenobarbital induction factor of CYP6D1, a cytochrome P450 monooxygenase, to autosome 2 (Liu and Scott 1997).

4.2 Materials and Methods

4.2.1 House fly Crosses and Phenotypes

Linkage analysis was performed by the association of gene polymorphisms (between aabys and OCR) with the five recessive mutant markers of the aabys strain

(Kozaki et al. 2002). Female aabys were crossed with male OCR to produce F_1 flies heterozygous for all five autosomes. The F_1 males were then backcrossed to aabys females. The offspring were sorted according to phenotype. Thirty-two phenotypes resulted, five of which were used to conduct the linkage analysis, being heterozygous at only one chromosome, as indicated by the absence of a recessive morphological marker. Flies that were heterozygous for one of each autosome (I-V) were denoted as *+/ac;ar/ar;bwb/bwb;ye/ye;snp/snp*, *ac/ac;+/ar;bwb/bwb;ye/ye;snp/snp*, *ac/ac;ar/ar;+/bwb;ye/ye;snp/snp*, *ac/ac;ar/ar;bwb/bwb;+/ye;snp/snp*, *ac/ac;ar/ar;bwb/bwb;ye/ye;+/snp*, respectively.

4.2.2 Genomic DNA extraction and sequence analysis

Genomic DNA was extracted from individual male house flies using the quick fly genomic DNA prep method (www.fruitfly.org). Briefly, a male fly was homogenized in 400 ml of buffer A (100 mM Tris-HCl, pH 7.5, 100 mM EDTA, 100 mM NaCl, and 0.5% SDS). The homogenate was incubated at 65°C for 30 min, followed by 10-min incubation on ice after being mixed with 0.8 ml of LiCl/KAc solution (4.3 M LiCl and 1.43 M KAc). The mixture was centrifuged at 14,000g for 15 min at 25°C. DNA was precipitated from the supernatant by addition of isopropanol, and then pelleted by centrifugation at 14,000g for 15 min at 25°C. The DNA pellet was washed with 70% ethanol and dissolved in 150 ml of TE buffer.

Sequences were sequenced at the Cornell Biotechnology Resource Center using an Applied Biosystems Automated 3730 DNA Analyzer. Sequences were aligned using the Lasergene MegAlign program (Clustal W method) and electropherograms were

analyzed using the Chromas program. Electropherogram figures were created using Sequencher.

4.2.3 *Mdα2* Polymorphisms

For *Mdα2*, genomic DNA was extracted from 12 aabys and 19 OCR parental individuals, 10 F_1 individuals, and at least three individuals from each previously stated backcross phenotype. Genomic DNA fragments (1,379 bp from aabys and 1,362 bp from OCR) were amplified using the Advantage® 2 polymerase mix (BD Bioscience Clontech) with a forward primer gM2VIIF1 (Table 4.1) and a reverse primer gM2VIIIR2 using the following thermal cycler program: 1 cycle of 95°C for 1 min, 30 cycles of 95°C for 30 s, 64°C for 30 s and 72°C for 2 min, and a final extension at 72°C for 7 min. The PCR product was purified using QIAquick PCR purification kit (Qiagen, Valencia, CA) and then sequenced using primer mdnachra2f2 (Table 4.1).

Table 4.1. Primer Sequences

Primer Name	Gene	Sequence (5'-3')
gM6F5	*Mdα6*	CAAGGCCGATGATGAGGCTGAGCT
gM6R4	*Mdα6*	CAGAACTGTCACTGTGGCTATTATTG
gM2VIIF1	*Mdα2*	GCACCTTGAGCGGCTACAAC
gM2VIIIR2	*Mdα2*	GACGGAGCCTCGCCCAGTATC
mdnachra2f2	*Mdα2*	AAGCAATCACGGCAAGGGCATC
gM5IF1	*Mdα5*	GAACCGCATTGTCACAAACCGCAC
5'M5Race8	*Mdα5*	GTGGTGGTGGTGGCAGCGATGGAG
MB3F0	*Mdβ3*	ACAATAATCTACGGCAGTCGGGTC
MB3R3	*Mdβ3*	ATCCTACACCGAATAGACAATGG
5'MBRace1	*Mdβ3*	GTACATCGAAGAGTAGCGTTGAAGTTGGA

4.2.4 *Mdα5* Polymorphisms

For *Mdα5*, genomic DNA was extracted from 10 aabys and 9 OCR parental individuals, 3 F_1 individuals, and 3 individuals from each of the five backcross phenotypes. Genomic DNA fragments (613 bp from aabys and 625 bp from OCR) were amplified using the Advantage® 2 polymerase mix (BD Bioscience Clontech) with a forward primer gM5IF1 and a reverse primer 5'M5Race8 (Table 4.1) using the following thermal cycler program: 1 cycle of 95°C for 1 min, 30 cycles of 95°C for 30 s, 64°C for 30 s and 72°C for 1 min, and a final extension at 72°C for 1 min. The PCR product was purified using QIAquick PCR purification kit (Qiagen, Valencia, CA) and then sequenced with the same primers used for amplification.

4.2.5 *Mdα6* Polymorphisms

Genomic DNA was extracted from 10 aabys and 9 OCR parental individuals, 11 F_1, and at least 3 individuals from each of the five backcross phenotype. A genomic DNA fragment from intron 10 (188-bp in aabys and 192-bp in OCR) was amplified using the 2x ReddyMix™ PCR master mix (ABgene House, Epsom, UK) with primers gM6F5 and gM6R4 (Table 4.1) using the following thermal cycler program: 1 cycle of 95°C for 1 min, 30 cycles of 95°C for 30s, 64°C for 30s and 72°C for 30s, and a final extension at 72°C for 7 min. The PCR product was purified using QIAquick PCR purification kit (Qiagen, Valencia, CA) followed by sequencing with the same primers used for amplification. When analyzing the backcross sequences, the reverse sequence was primarily used since several insertions/deletions in the alleles made it relatively easier. For this reason, the reverse electropherograms are presented in analysis.

4.2.6 *Mdβ3* Polymorphisms

Genomic DNA was extracted from 1 aabys and 1 OCR parental individual. For this analysis, most of the gene was first amplified using forward primer MB3F0 and reverse primer MB3R3 (Table 4.1) and Advantage® 2 polymerase mix (BD Bioscience Clontech) with the following thermal cycler program: 1 cycle of 95°C for 2 min, 30 cycles of 95°C for 30s, 64°C for 30s and 72°C for 2 min, and a final extension at 72°C for 5 min. The PCR product was purified using QIAquick PCR purification kit (Qiagen, Valencia, CA) followed by sequencing using the same primers used for amplification as well as an additional primer 5'MBRace1, which started in the middle of the gene so as to obtain the full gene sequence.

4.3 Results and Discussion

4.3.1 Linkage of *Mdα2*

Two alleles in aabys were identified, denoted as A (accession no. DQ393143) and B (accession no. DQ393144), and two alleles in OCR were identified, denoted as B (accession no. DQ393145) and C (accession no. DQ372064). Their alignment report can be viewed in Figure 4.1. Allele A was unique to the aabys strain and allele C was unique to the OCR strain. Allele A was the most common in aabys flies, whereas allele C was the most common in OCR flies (Table 4.2). When determining which autosome *Mdα2* was on, I looked for the one phenotype, out of the five isolated, that gave us individuals heterozygous for *Mdα2*. Since both strains shared a common allele, allele B, I could not count the genotype AB as definitively heterozygous, nor the genotype BC.

Only the presence of the AA genotype reliably indicated that the individual was homozygous, and could not be heterozygous at the gene of interest. The genotype CC would not be found, since aabys was used for the backcross. Genotype AA was present in the phenotypes heterozygous for autosome 1, 3, 4 and 5. No AA genotype was found in those heterozygous for autosome 2 (ac/ac;+/ar;bwb/bwb;ye/ye;snp/snp). Additional individuals from this phenotype were genotyped to confirm the absence of AA individuals. Not only this, but the OCR allele C was found only in individuals heterozygous for autosome 2 (Table 4.3). These results indicate that *Mdα2* is present on autosome 2 (Table 4.4), which is consistent with *Drosophila/Musca* homology maps (Foster et al., 1981).

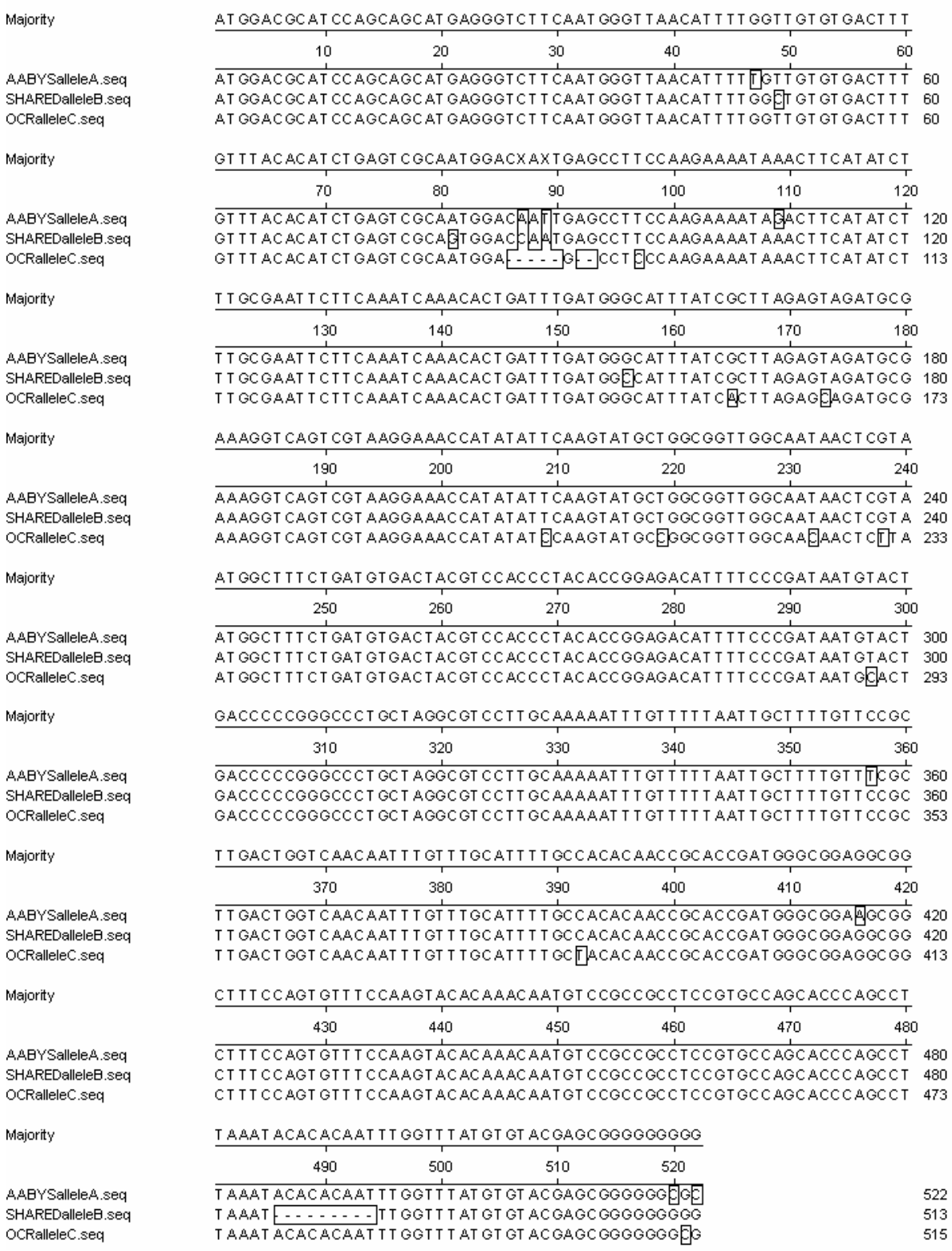

Figure 4.1. Alignment report of the three alleles found for *Mdα2*. The boxed regions are areas of difference between the alleles.

Table 4.2. *Mdα2* allele summary.

Allele	Strain	Frequency	Accession Number
A	aabys	0.833	DQ393143
B	aabys	0.167	DQ393144
B	OCR	0.132	DQ393145
C	OCR	0.868	DQ372064

Table 4.3. Observed frequencies of the various *Mdα2* genotypes in backcross (♀ aabys x ♂ F_1 (♀ aabys x ♂ OCR)) individuals.

	Mdα2 alleles				
Phenotype	AA	AB	BB	CA	BC
+;ar;bwb;ye;snp (n=3)	1.00	0	0	0	0
ac;ar;+;ye;snp (n=3)	1.00	0	0	0	0
ac;ar;bwb;+;snp (n=3)	0.67	0.33	0	0	0
ac;ar;bwb;ye;+ (n=3)	1.00	0	0	0	0
ac;+;bwb;ye;snp (n=10)	0	0.1	0.1	0.5	0.3

Table 4.4. Linkage analysis for *Mdα2*.

Phenotype	Number of individuals homozygous for A allele
+;ar;bwb;ye;snp	3/3
ac;+;bwb;ye;snp	0/10
ac;ar;+;ye;snp	3/3
ac;ar;bwb;+;snp	2/3
ac;ar;bwb;ye;+	3/3

4.3.2 Linkage of *Mdα5*

One allele was identified in each strain. The aabys strain had the unique allele D (accession no. EF203214), and the OCR strain had the unique allele E (accession no. EF203215), thus the frequency of these alleles in their respective strain was 1. The alleles had several sites that differed from each other, as exemplified by the alignment report in figure 4.2. The resulting sample electropherograms for F_1 and backcross individuals, Figures 4.3, 4.4, 4.5, offered several reliable polymorphisms that differentiate between a heterozygote (DE) and homozygote (DD). When examining the backcross phenotypes, it was found that only individuals heterozygous for autosome 1 (*+/ac;ar/ar;bwb/bwb;ye/ye;snp/snp*) were also heterozygous for Mdα5, with the genotype DE (Table 4.5). All other backcross phenotypes were homozygous for the aabys allele D. This is evident in the electropherograms, where the *+/ac;ar/ar;bwb/bwb;ye/ye;snp/snp* (Figure 4.4) individual resembles the heterozygous F_1 individual (Figure 4.3) and the other backcross individuals (Figure 4.5) are all homozygous. This indicates that *Mdα5* is located on autosome 1.

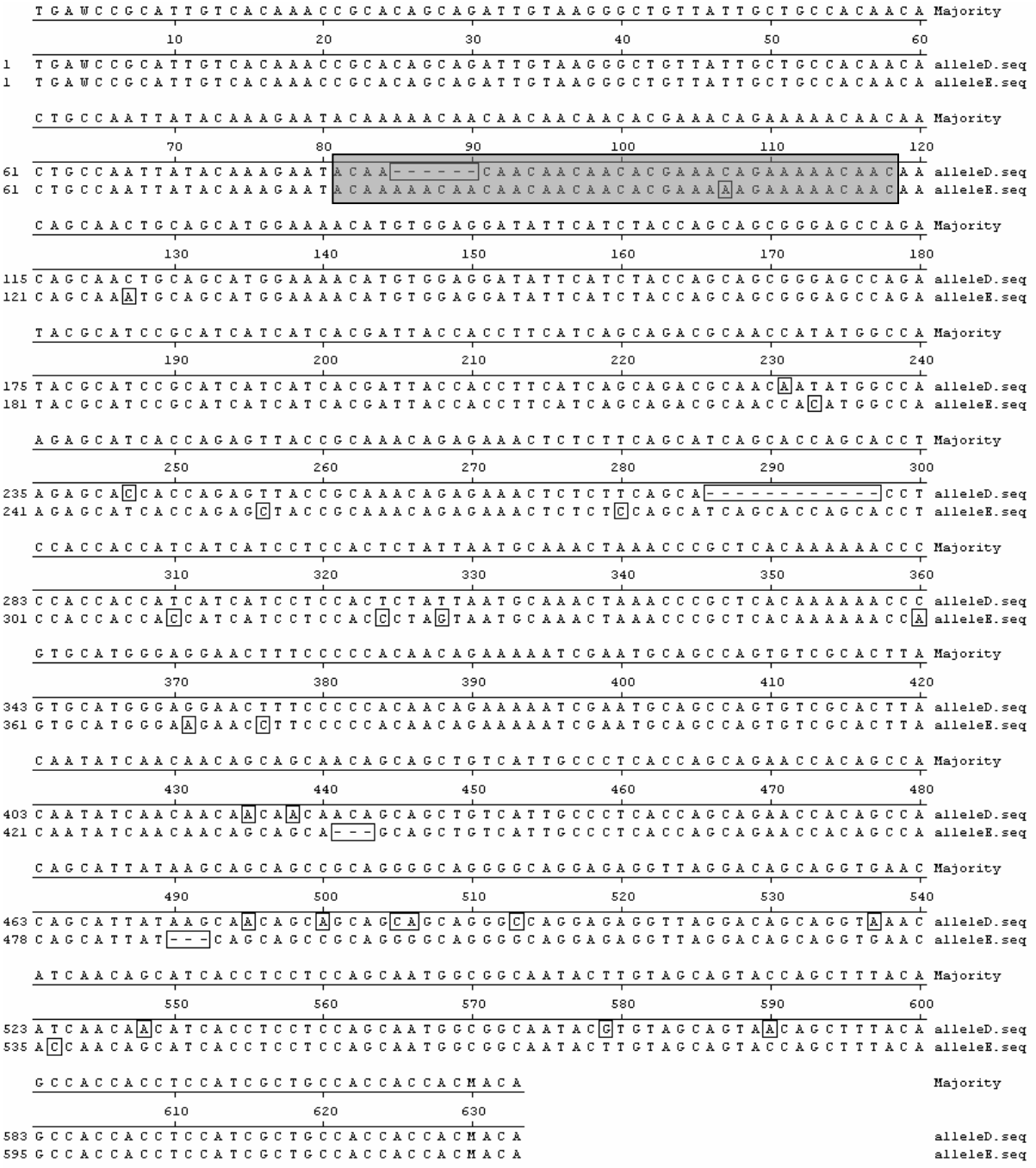

Figure 4.2. Alignment report for allele D (aabys) and allele E (OCR) in *Mdα5*. The boxes indicate differences between the two alleles. The grey shaded region represents the area illustrated by the electropherograms in figures 4.3, 4.4, and 4.5.

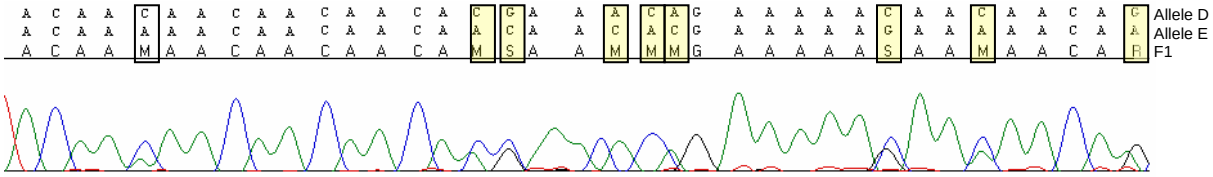

Figure 4.3. Representative electropherogram segment of a forward sequence of an F_1 (♀ aabys x ♂ OCR) individual. Yellow boxes indicate polymorphisms used to assess genotype.

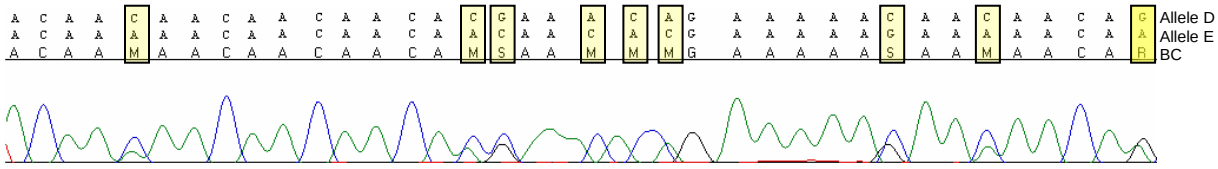

Figure 4.4. Representative electropherogram of a forward sequence of a backcross individual with +;ar;bwb;ye;snp phenotype. Yellow boxes indicate polymorphisms used to assess genotype.

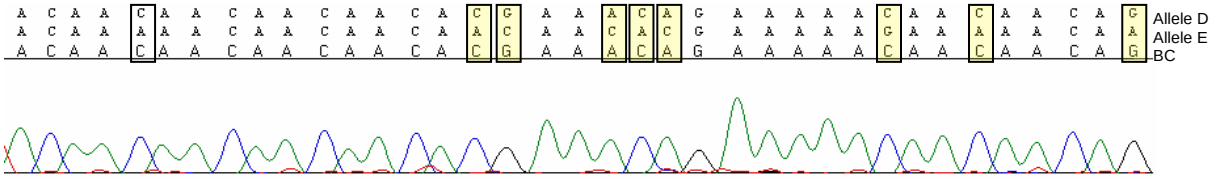

Figure 4.5. Representative electropherogram of a forward sequence of a backcross individuals with ac;+;bwb;ye;snp, ac;ar;+;ye;snp, ac;ar;bwb;+;snp and ac;ar;bwb;ye;+ phenotypes. Yellow boxes indicate polymorphisms used to assess genotype.

Table 4.5. Linkage analysis for *Mdα5*.

	Individuals with alleles	
Phenotypes	DE	DD
+;ar;bwb;ye;snp	3/3	0/3
ac;+;bwb;ye;snp	0/3	3/3
ac;ar;+;ye;snp	0/3	3/3
ac;ar;bwb;+;snp	0/3	3/3
ac;ar;bwb;ye;+	0/3	3/3

4.3.3 Linkage of *Mdα6*

Two alleles were found in the aabys strain, denoted as allele F (accession no. DQ498139) and G (accession no. DQ498140) at a frequency of 0.79 and 0.21, respectively. One allele was found in the OCR strain, denoted as H (accession no. DQ498141). Their sequences and alignment can be viewed in Figure 4.6. This resulted in two heterozygous possibilities for F_1s (FH and GH, only FH observed, Figure 4.7). In the backcross individuals, three heterozygotes were possible (FH, GH and FG), two of which were observed. The FH (observed, Figure 4.8) and GH (not observed) genotypes would be the result of an aabys and OCR allele, whereas the FG genotype (observed, Figure 4.10) would be a result of the two aabys alleles. Two homozygotes were possible, FF and GG, one of which was observed (Table 4.6, Figure 4.9). Both homozygotes would be the result of two aabys alleles. Homozygotes of OCR alleles could not be observed since the aabys and OCR F_1 was backcrossed to aabys, not OCR. Genotyping the five backcross phenotypes resulted in allele H only being present in individuals heterozygous for autosome 1 (+/ac;ar/ar;bwb/bwb;ye/ye;snp/snp) (Table 4.7). This indicates that *Mdα6* is present on autosome 1. Recent studies on the homologous subunit gene, *Dα6* in *D. melanogaster*, indicated that a mutation in this subunit would be sufficient to confer high levels of spinosad resistance (Perry et al. 2007a), but data on resistance in the house fly has shown that although *Mdα6* is located on autosome 1, it is not responsible for resistance in laboratory strains (Gao et al. 2007b).

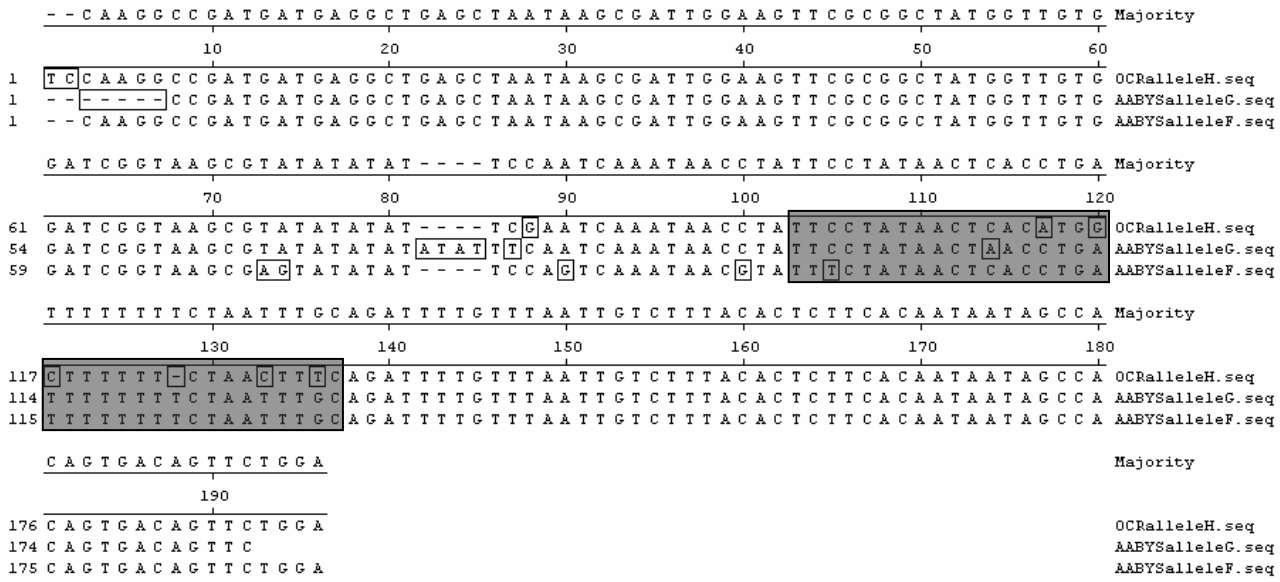

Figure 4.6. Alignment report of alleles H, F and G. The boxes indicate differences between the three alleles. The grey shaded region represents the area illustrated by the electropherograms in figures 4.7, 4.8, 4.9, and 4.10.

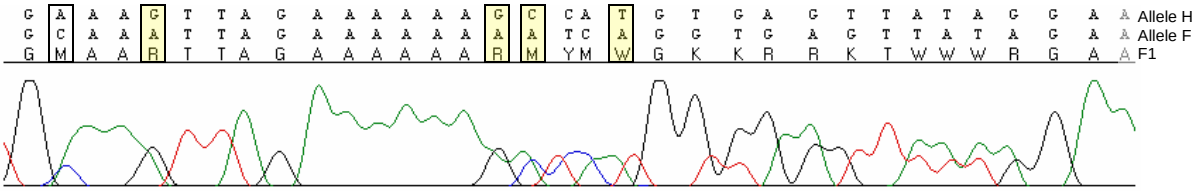

Figure 4.7. Representative electropherogram segment of a reverse sequence of an F_1 (♀ aabys x ♂ OCR) individual. Yellow boxes indicate polymorphisms unique only to a heterozygote containing the OCR H allele. The genotype is FH.

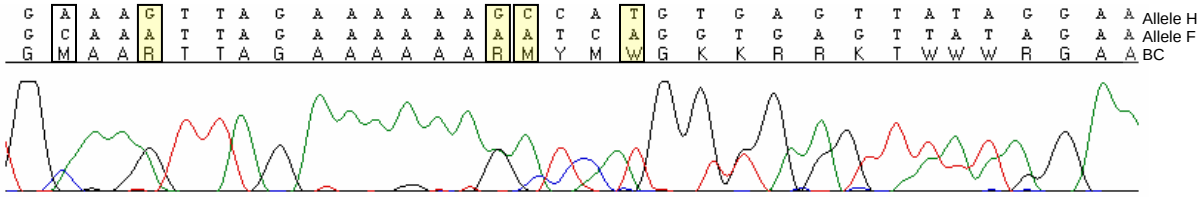

Figure 4.8. Representative electropherogram segment of a reverse sequence of a backcross individual with +;ar;bwb;ye;snp phenotype. Yellow boxes indicate polymorphisms unique only to a heterozygote containing the OCR H allele. The genotype is FH.

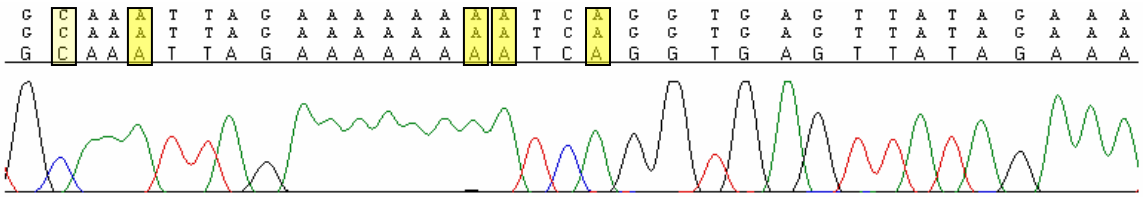

Figure 4.9. Representative electropherogram segment of a reverse sequence of a backcross individuals with ac;+;bwb;ye;snp, ac;ar;+;ye;snp, ac;ar;bwb;+;snp and ac;ar;bwb;ye;+ phenotypes. Yellow boxes indicate areas where a nucleotide unique to the OCR H allele is absent. The genotype is FF.

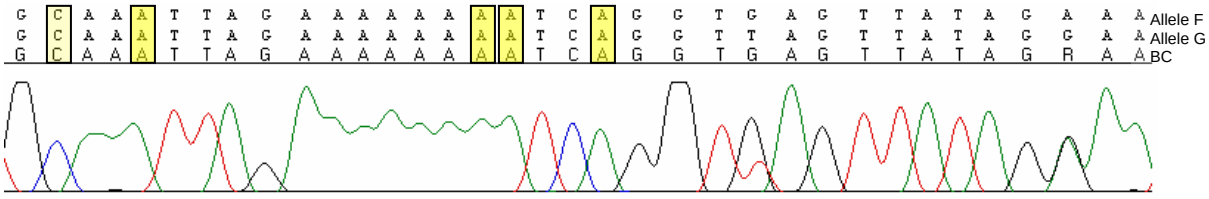

Figure 4.10. Representative electropherogram segment of a reverse sequence of a backcross individuals with ac;+;bwb;ye;snp, ac;ar;bwb;+;snp and ac;ar;bwb;ye;+ phenotypes. Yellow boxes indicate areas where a nucleotide unique to the OCR H allele is absent. The genotype is FG, and although it is a heterozygote, it is a variant completely derived from aabys.

Table 4.6. Summary of observed genotypes for each phenotype. Note the absence of the *Mdα6* H allele in individuals heterozygous for autosomes 2,3,4 and 5.

	Individuals with alleles				
Phenotypes	FH	GH	FG	FF	GG
+;ar;bwb;ye;snp	3/3				
ac;+;bwb;ye;snp			1/3	2/3	
ac;ar;+;ye;snp				3/3	
ac;ar;bwb;+;snp			3/5	2/5	
ac;ar;bwb;ye;+			1/6	5/6	

Table 4.7. Linkage Analysis for *Mdα6*.

Phenotype	Number of individuals with *Mdα6* H allele
+;ar;bwb;ye;snp	3/3
ac;+;bwb;ye;snp	0/3
ac;ar;+;ye;snp	0/3
ac;ar;bwb;+;snp	0/5
ac;ar;bwb;ye;+	0/6

4.3.4 Linkage of *Mdβ3*

Analysis of *Mdβ3* revealed that it does not contain any introns (Gao et al. 2007). Regardless, the gene was sequenced and examined for polymorphisms between aabys and OCR in the exon region. Two alleles were found, alleles J (accession no. EF203216) and K (accession no. EF203221), but neither was unique to aabys nor OCR. Their sequences and alignment can be viewed in Figure 4.11. No reliable polymorphisms were found between the aabys and OCR strains, making it impossible to determine the linkage analysis with this sequence information.

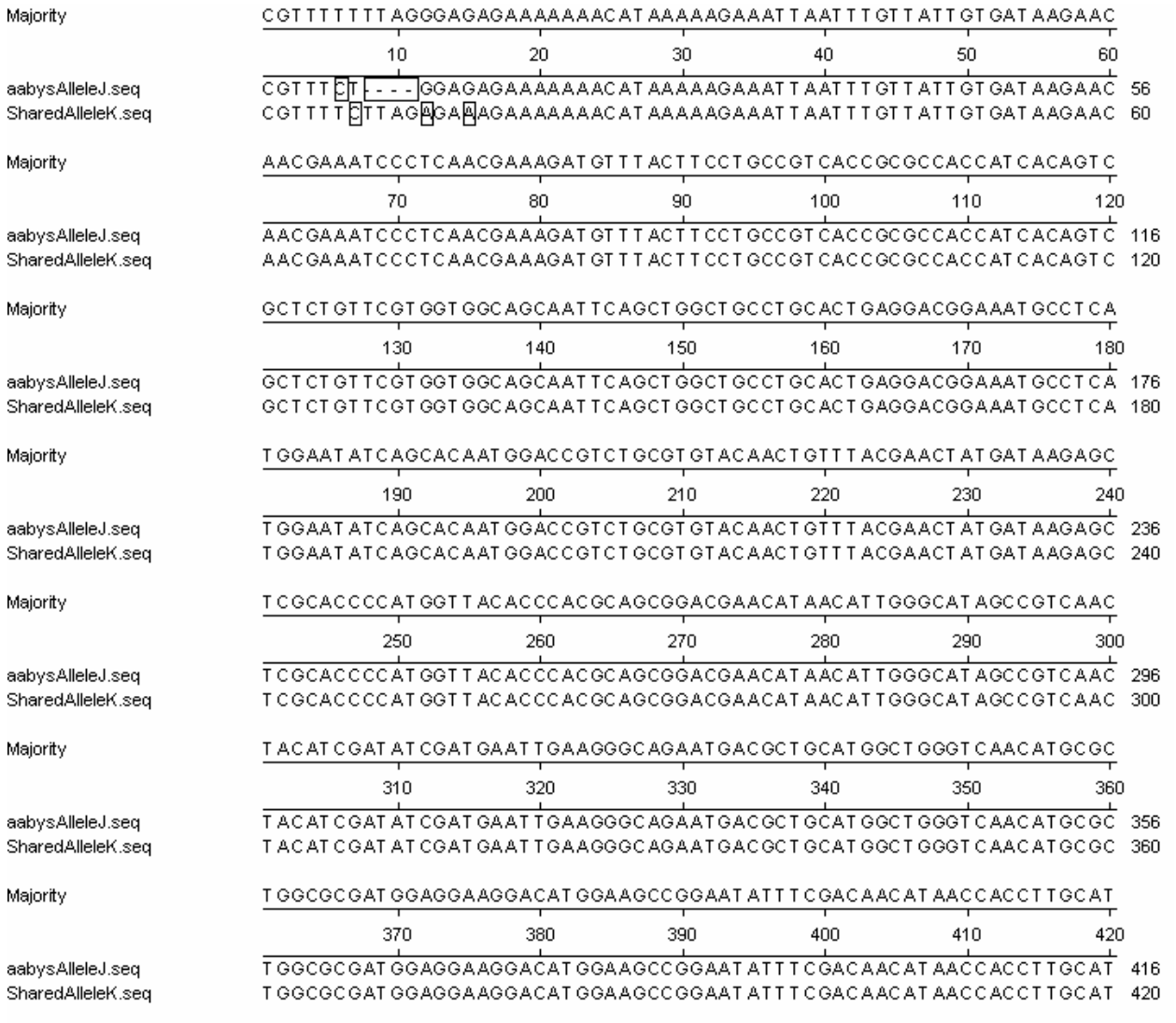

Figure 4.11. Alignment report of alleles J, from aabys, and K, from both aabys and OCR. Boxes indicate areas that differ between the two alleles.

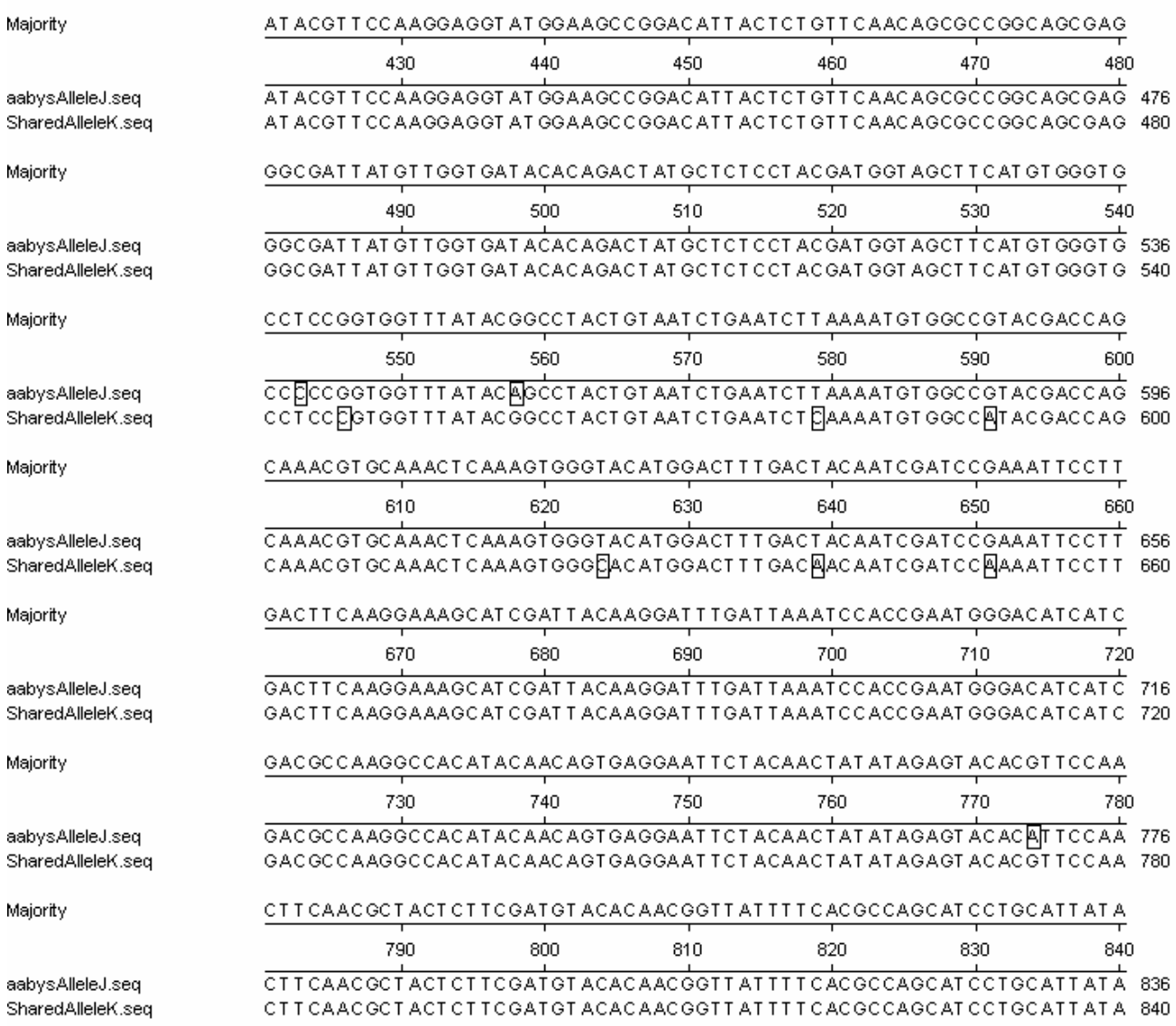

Figure 4.11. Continued.

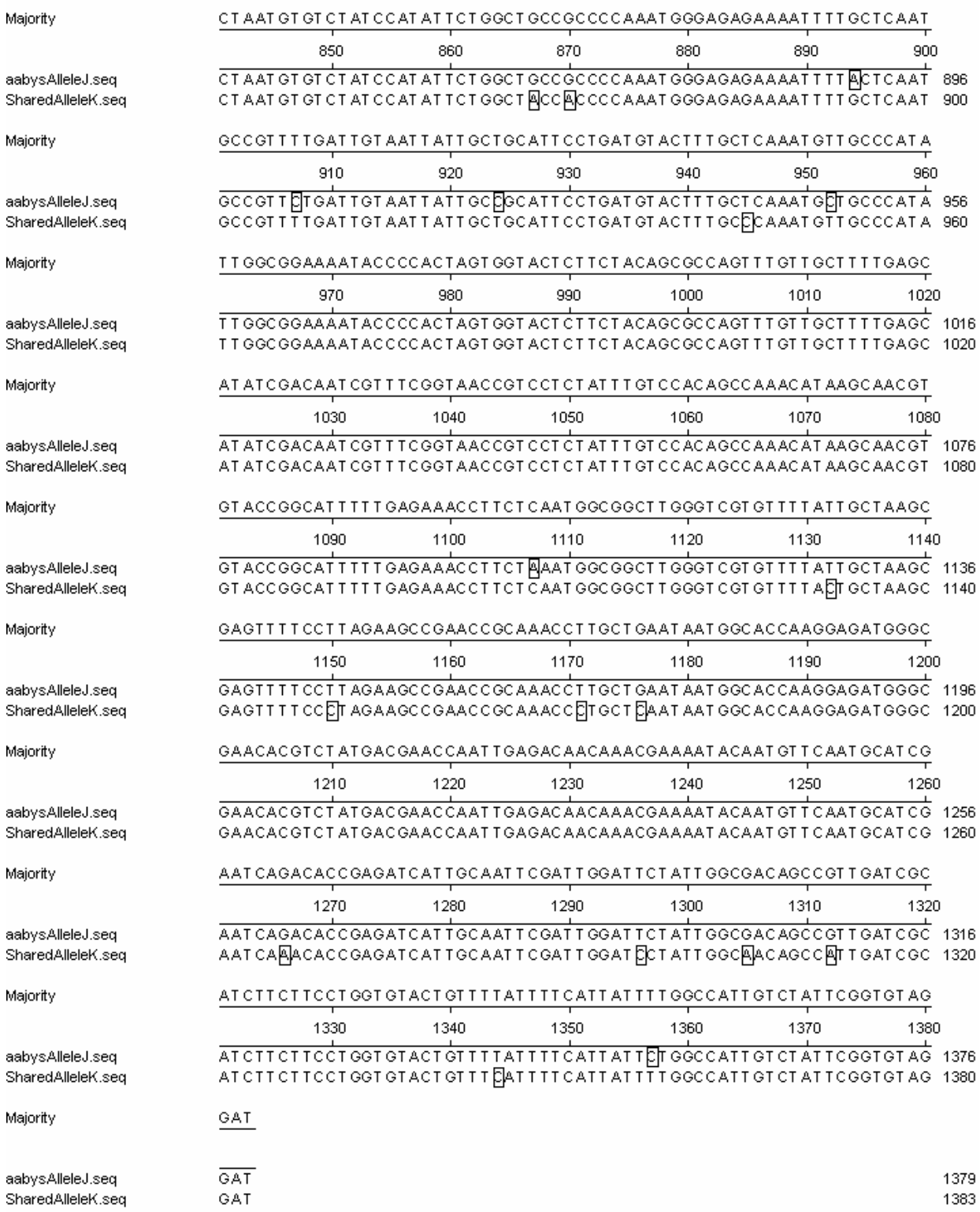

Figure 4.11. Continued

Chapter 5: Mating Competition

5.1 Introduction

The evolution of a population of organisms to some selective pressure usually comes with a fitness cost, such that the evolved population is less fit in its previous environment (Fisher 1958). Over the past few decades, resistance to insecticide has been documented as having fitness costs, which may include reduced reproductive success (Ferrari and Georghiou 1981). Reproductive disadvantages in a number of insects, such as reduced paternity (Higginson et al. 2005) and reduced ability to compete against susceptible males (Berticat et al. 2002), have been documented.

Understanding the fitness effects of spinosad resistance is an important component of understanding how the resistance may progress in the field. This study focused on the fitness effects of spinosad insecticide resistance in the house fly, *M. domestica* L.. We examined a key fitness component, mating success, using two methods.

One method used visual observation to determine whether individual female flies mated with a resistant male or susceptible male. Since females typically mate only once (Riemann et al. 1967), the first male seen mating with her will likely be the only male she mates with and thus sire her offspring. The other method used a spinosad bioassay to determine whether a mated female's offspring were resistant or susceptible, and from this deduce which male she mated with.

5.2 Materials and Methods

Two house fly strains were used, aabys, a susceptible strain with recessive mutant markers, and rspin, a spinosad resistant strain with recessive mutant markers, which aside from the resistance, is highly related to aabys (Shono and Scott 2003). Both strains were reared under identical conditions to help maintain uniformity in size.

House fly larvae were reared on medium containing 2.3 liters of water, 0.5 kg calf manna (Manna Pro Corp, St. Louis, MO), 90 g bird and reptile little wood chips (Northeastern Products Corp, Warnersburg, NY), 0.8 kg wheat bran (Agway; Ithaca, NY), and 50 g dried active baker's yeast (ICN Biomedicals, Costa Mesa, CA). The adults were raised on powdered milk + white granulated sugar (1:1 ratio by volume) and water, *ad libitum*.

5.2.1 Mating competition assays: Visual observation method

When adult flies began to emerge, the containers were cleared of any flies and placed in a 16°C chamber overnight. The flies were then allowed to warm and emerge over a period of time not greater than 6 hours. The virgin flies were sorted by gender and kept in the biochamber until ready to assay.

Flies were assayed from 4-6 days old. Male flies were painted with a dot of Sharpie "paint" (red or silver) on the dorsal part of their thorax at least 1 day prior to mating competition assay. Only flies with fully expanded wings were used. On the day of the assay, one female (either rspin or aabys) was paired with a painted rspin and aabys male. The two males were paired according to size, with their sizes being as similar as possible in order to eliminate size as a potential factor in mating competition. To control

for paint color, reciprocal pairings were made. Both aabys and rspin females were matched with a pair of males. The trio was placed in a 20 ml scintillation vial topped with bridal veil secured with a rubber band and observed every fifteen minutes for at least three hours. The flies typically mated for longer than fifteen minutes, so if a mating occurred, it was observed. The vials were initially kept in a humid box, but this was not enough to slow dehydration so in later trials the opening of the vial was plugged with cotton moistened with water and wrapped in bridal veil. The vials were placed under a lamp during observation and a pair of flies was scored as mating only if the female's ovipositor was drawn into the male.

5.2.2 Mating competition assays: Spinosad topical bioassay method

To ensure female virginity, emerging flies were sorted by gender every six hours. Approximately 200 each of rspin males, rspin females and aabys males were collected. These were kept in separate containers with food and water for two days, and labeled by day of emergence and gender.

On the second day after emergence, individual flies were weighed. A collection of 15 rspin males were compared with 15 aabys males. Pairs of males were assigned based on their closeness in weight. Each pair of males was placed into a cup with a small amount of food and a scintillation vial filled with distilled water and a cotton wick. Rspin females were also weighed, and placed in a cup with the two males, although their weight was not aligned with that of the males. Each group of 15 cups was considered a set, and 4 sets (A, B, C and D) were assembled over 3 days. A total of 63 trios were assembled (18 trios in set A).

Each trio of flies was left undisturbed for three days. On the third day, the female was removed and placed in a paper drinking cup with media. She was allowed to lay eggs on the media for three days, or until she died, whichever came sooner.

The offspring from these cups were allowed to emerge into larger containers that held food and water. Both males and females were topically assayed with spinosad when they were 3-5 days old. In the assays, 20 flies, or as many as had emerged, were placed in a 180 ml Sweetheart waxed paper cup with food. A diagnostic dose of 2.50 $x10^{-1}$µg of spinosad per fly was topically applied to the fly's thoracic notum using 0.5µl of 0.500mg/ml acetone solution. The cup was covered in bridal veil and water soaked cotton was placed on top. Flies were held at 25°C. Mortality was assessed after 24 hrs with flies that were ataxic being scored as dead**.**

5.3 Results and Discussion

5.3.1 Mating competition assays: Visual observation method

Initial experiments showed that assaying the flies at a consistent age is very important. Delaying the emergence using chilling techniques affected the willingness to mate. The flies must be assayed at 5 days of age. Flies used at 4 and 6 days did not mate. Other laboratory studies have found that wild type flies will readily mate at these ages (unpublished data), so this high sensitivity to age is likely due to the strain being mutant and highly inbred. The paint did not appear to have an impact on the flies' willingness to mate, based from the paint vs. no paint trial.

Sixteen trios mated out of 43 when flies were aged 5 days (Table 5.1). According to Fisher's Exact Test (P = 0.49) there were no differences in mating ability. The flies were not very responsive, however, and were all part of the same trial conducted on one day, so further trials are needed. Part of the reason for the low responsiveness is that the flies began to suffer from dehydration after about 2 hours.

In order to confirm or reject a mating fitness cost associated with spinosad resistance using this method, further trials will be needed. To conduct further trials, a strict procedure will need to be developed, as the flies are extremely sensitive to age variables, dehydration and other unknown factors. When assayed, the flies must be 5 days old and must be provided with a water source. These complications prompted the spinosad topical bioassay method, which avoids many post-emergence variables.

5.3.2 Mating competition assays: Spinosad topical bioassay method

This method was developed in order to avoid variables that hindered the previous experiment. It has the added benefit of more accurately predicting mating success through the measure of offspring production, as mating may not necessarily predict fertilization or offspring survivorship. Also, the flies were given a couple days to mate, which is much more realistic than the few hour window of time in the observational experiment.

I was confident that using a topical bioassay on the F_1 offspring would accurately indicate whether the father was rspin or aabys. Previous experiments with topical spinosad bioassays have shown that there is enough resolution between homozygous resistant (RR) and heterozygous (SR) to accurately determine which male a female house

fly mated with (Deacutis et al. 2007). Since spinosad is a recessive trait (Shono and Scott 2003), the experiment was conducted using only the resistant, rspin females so that the possible resulting offspring were either SR or RR. Using susceptible females would result in SS offspring, which would be difficult to discriminate from SR offspring when using a topical spinosad assay. Therefore, the reciprocal cross was not conducted. In the following discussion, "n" refers to the number of flies and "N" refers to the number of repetitions.

Out of the 63 trios of flies (rspin ♀, rspin ♂, aabys ♂), 55 females were put on media cups. This number decreased as some flies in the trio died over the three days. F_1 offspring were obtained from 39 out of the 50 cups. Out of the thirty-nine emerged cups, thirty-two were used in data analysis (N=32). Only cups that produced more than 10 offspring, and were clearly either SR or RR, were used. Also cups with offspring that were not clearly SR or RR were not used, which excluded three cups. Cups were scored as having SR offspring if their mortality ranged between 75-100%, and as RR if it ranged from 0-25%. Most cups clearly fell within one of these two ranges (Table 5.2). Mortality for the rspin flies was 2.5% (± 0.54, n= 120, N= 6), and 99.7% (± 0.32, n = 80, N= 4) for the rspin x aabys F_1 comparison group. Even though the F_1 mortality was very consistent, it was necessary to choose broader ranges to tally the experimental group, as low emergence and thus small sample size increased the variability. Final tallies indicated that 7/32 cups contained offspring of an rspin male, and 25/32 cups contained offspring of an aabys male. A chi-square analysis demonstrates that aabys males have a significant mating advantage (P = 0.0015). This experiment should be repeated to confirm the results.

Although mating competition is one possible factor, another possible factor, which is not excluded by this experiment, is offspring viability. Offspring were obtained from 39 out of 50 possible cups, and even though 100% emergence is not expected, the design of the experiment makes it impossible to assess why each of the 11 cups did not produce offspring. It is therefore possible that males from one strain generate less viable offspring. A simple experiment comparing offspring viability of the two strains would resolve this question. Also, non-mutant resistant and susceptible isogenic strains can be created, which may increase the overall cup emergence, and the mating competition can be repeated.

Table 5.1. Assay conducted on 5 day old flies. Forty-three trios assembled, sixteen matings observed.

		Female	
		aabys	rspin
Male	aabys	1	4
	rspin	2	9

Table 5.2. Bioassay mortality data from the offspring of the mating competition trio. Each cup represents offspring from an rspin (RR) female that chose to mate with either an rspin (RR) male or aabys (SS) male.

Cup number	Number of flies tested	% Mortality	SR/RR
A1	14	100	SR
A2	25	12	RR
A3	68	15	RR
A5	78	100	SR
A6	57	5	RR
A8	14	29	?
A10	77	99	SR
A12	40	5	RR
A13	58	10	RR
A15	114	98	SR
A16	24	100	SR
B2	15	100	SR
B3	16	100	SR
B5	36	100	SR
B6	11	100	SR
B7	99	100	SR
B10	38	100	SR
B12	14	100	SR
B13	26	96	SR
B15	22	100	SR
C1	81	99	SR
C2	47	98	SR

C4	17	100	SR
C5	42	100	SR
C6	31	100	SR
C7	44	100	SR
C8	20	55	?
C13	51	98	SR
C15	18	22	RR
D2	40	100	SR
D4	11	18	RR
D9	17	94	SR
D11	12	83	SR
D13	12	50	?
D14	20	100	SR

Table 5.2. Continued.

Chapter 6: Future Directions

The resistance monitoring study (Chapter 3) unveiled some of the possible complications of a resistance monitoring program using purely insecticidal bioassays. These are namely the facts that susceptibility varied greatly from site to site, and thus establishing baseline data to characterize all farms would be difficult. Also, since resistance is highly recessive, heterozygotes will not be readily detected. Of great interest is to develop a sensitive resistance monitoring assay that will be able to detect the presence of heterozygotes. This type of assay may require molecular assays, and the identification of the gene responsible for resistance.

One possibility for examining the genetic basis for resistance would be to create nAChR gene knockouts, as done in *D. melanogaster* (Perry et al. 2007b). It would be interesting to see whether *Mdα6* knockout house flies attain resistance, as in *D. melanogaster*. Although this gene does not confer resistance in laboratory strains, it is possible that a mutation may confer a different type of resistance. Doing this may give better insight as to which subunits are crucial for spinosad mode of action.

The fitness costs to spinosad must also be explored in further depth. First of all, non-mutant isogenic resistant and susceptible house fly strains should be established. These strains will be more robust than the mutant animals, and thus be better subjects in fitness studies. Establishing such strains will also allow fitness studies that are more realistic to field conditions. Observational mating competition, such as in section 5.2.1, will most likely be more successful with such strains. Other measures of fitness can also be examined, such as overwintering and fecundity.

Chapter 7: References

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