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Multiplicative interaction between the two major mechanisms of permethrin resistance, *kdr* and cytochrome P450-monooxygenase detoxification, in mosquitoes

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Abstract

Epistasis is the nonadditive interaction between different loci which contribute to a phenotype. Epistasis between independent loci conferring insecticide resistance is important to investigate as this phenomenon can shape the rate that resistance evolves and can dictate the level of resistance in the field. The evolution of insecticide resistance in mosquitoes is a growing and world-wide problem. The two major mechanisms that confer resistance to permethrin in *Culex* mosquitoes are target site insensitivity (i.e. *kdr*) and enhanced detoxification by cytochrome P450 monooxygenases. Using three strains of mosquitoes, and crosses between these strains, we assessed the relative contribution of the two independent loci conferring permethrin resistance, individually and when present together. We found that for all genotype combinations tested, *Culex pipiens quinquefasciatus* exhibited multiplicative interactions between *kdr* and P450 detoxification, whether the resistance alleles were homozygous or heterozygous. These results provide a basis for further analysis of the evolution and maintenance of insecticide resistance in mosquitoes.

Introduction

Epistasis is the nonadditive interaction between different loci which contribute to a phenotype (Cheverud & Routman, 1995; Moore & Williams, 2005; Hartl & Clark, 2007). Independent loci conferring insecticide resistance can exhibit joint action in a less than additive manner (i.e. antagonistic epistasis), an additive manner, or in a greater than additive manner (i.e. synergistic epistasis) (Scott *et al.*, 1984). Evolutionarily, epistasis favours natural selection to purge populations of deleterious mutations (Kitagawa, 1967; Mukai, 1969; Chao, 1988), affects changes in allele frequency (Karlin, 1975), helps maintain equilibrium but with increased genetic variation decreasing the problem of genetic load (Gavrilets & Jong, 1993), and alters fitness effects of a genotype (Karlin, 1975).

Studies examining the presence or absence of epistasis between multiple loci have been conducted on an assortment of organisms including bacteria, fungi (de Visser *et al.*, 1997), plants (Yu *et al.*, 1997) and animals (Spassky *et al.*, 1965). In general, these studies have found that the type of epistasis (or lack thereof) between independent loci is dependent on the environment (Blows & Hoffmann, 1996; Bohannan *et al.*, 1999; Clauss *et al.*, 2006) in which the organism evolves, the phenotype measured (Doebley *et al.*, 1995; Clauss *et al.*, 2006), and the genetic backgrounds (Doebley *et al.*, 1995; Elena & Lenski, 2001; Garner *et al.*, 2005) of the organism. For example, examination of fitness costs associated with multiple resistance mechanisms present in *Escherichia coli* to bacteriophages showed that epistasis played a role in decreasing the total cost associated with the mechanisms in a trehalose rich environment vs. a glucose environment (Bohannan *et al.*, 1999). In *Arabidopsis*, synergistic epistasis occurs only between the phenotypes trichome density and indole glucosinolate concentration (unlinked plant resistance mechanisms to herbivory) in a field experiment when general herbivory is measured (Clauss *et al.*, 2006). In human dizygotic twin pairs, those with the *ibs*C variable, compared to those with the *ibs*T variable, showed significance of an

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interaction between the *Xmn*I-Gγ genotype and linkage to a region on chromosome 8, which influences the expression of foetal haemoglobin (Garner *et al.*, 2005).

Like a variety of phenotypes in many organisms, the evolution of insecticide resistance in a population results from the balance between the fitness advantage resistance alleles confer in the presence of insecticides and their fitness cost in the absence of insecticides. In a population with multiple resistance mechanisms, the evolutionary outcome of each is additionally shaped by the relative interaction of the resistance alleles in both environments. Therefore, to fully assess the evolution of resistance, the type of interaction between the resistance loci, the fitness costs and the interplay between these components must be considered.

Although it is difficult to evaluate the interactions between fitness costs of resistance alleles in the absence of insecticide exposure in field populations (because of unknown alleles, fitness epistasis, pleiotropic genes, etc.), insecticide bioassays can be used to evaluate if there is an interaction between resistance loci in the presence of insecticides (using strains with known mechanisms). It would be expected that an interaction between resistance loci that provided protection greater than either loci alone would be favoured in the presence of insecticides.

Given that there are a limited number of mechanisms responsible for insecticide resistance, it might be possible to establish general rules for interactions between loci that would be useful in the development of resistance management strategies. Studies of the interactions between resistance loci have been most commonly conducted in house flies (Georghiou, 1969; Sawicki, 1970; Scott *et al.*, 1984; Takada *et al.*, 1988; Lee & Shono, 1996; Park *et al.*, 1996; Zhang *et al.*, 1997; Shono *et al.*, 2002, 2004). Generally, a greater than additive interaction is observed between two loci that are both homozygous resistant, whereas additivity (lack of epistasis) occurs between two loci that are both heterozygous. A complication to understanding interactions between resistance mechanisms is that homozygous and heterozygous resistant individuals exist in field populations (Rinkevich *et al.*, 2006) and often confer differing levels of resistance. Thus, it is desirable to know the type of interaction occurring between resistance loci when they are present in both homozygous and heterozygous conditions. Herein, we examined the interaction of two pyrethroid resistance loci in both the homozygous and heterozygous state.

Insecticide resistance is a significant problem that limits control of medically important pests and is extremely detrimental in the on-going struggle to control or eradicate vectors of diseases (WHO, 1976). Mosquitoes are capable of transmitting a multitude of pathogens that cause deadly and debilitating human and animal diseases. *Culex pipiens quinquefasciatus*, the southern house mosquito, is the primary vector of the filarial nematode, *Wuchereria bancrofti*, which causes filariasis (Vinogradova, 2000). This species is also a competent vector of West Nile virus (WNV) (Turell *et al.*, 2001; CDC, 2002), St Louis encephalitis in eastern United States (Eldridge *et al.*, 2000), avian pox virus, bird malaria pathogens and dog heartworm (*Dirofilaria immitis*). Mosquito population control is primarily achieved through the use of pyrethroid, organophosphate and carbamate insecticides (Zaim *et al.*, 2000; Cui *et al.*, 2006a; WHO, 2006). Reports of resistance to pyrethroids in larvae of the *Culex pipiens* complex have been recorded worldwide (Rodriguez *et al.*, 1993; Ben Cheikh *et al.*, 1998; Bisset & Soca, 1998; Chandre *et al.*, 1998; Kolaczinski & Curtis, 2004; Liu *et al.*, 2004; Paul *et al.*, 2004; Yebakima *et al.*, 2004; Xu *et al.*, 2005; Cui *et al.*, 2006b).

The two major mechanisms that confer resistance to pyrethroids in *Culex* mosquitoes are target site insensitivity (Martinez-Torres *et al.*, 1999) and enhanced detoxification by cytochrome P450 monooxygenases (Kasai *et al.*, 1998). The decrease in sensitivity of the insect nervous system to DDT and pyrethroid insecticides is due to a L1014F mutation (called *kdr*) in the voltage sensitive sodium channel (*Vssc*) (Williamson *et al.*, 1996; Martinez-Torres *et al.*, 1999). Cytochrome P450 monooxygenases (P450s) are an important biochemical system involved in the metabolism of xenobiotics (such as insecticides) and endogenous compounds (such as hormones) (Agosin, 1985; Gilbert, 2004; Rewitz *et al.*, 2006). Insect P450s are important because they can detoxify (i.e. limit the toxicity) and bioactivate (i.e. produce more toxic metabolites) insecticide compounds (Agosin, 1985; Scott, 1999). Determining if an interaction is present between loci of resistance is important to investigate because it may play a role in the evolution and maintenance of multigenic resistance in the field, consequently dictating the effectiveness of a chemical control campaign.

In this study, we determined if and how *kdr* interacted with a P450-mediated resistance (detoxification) locus in *Cx. p. quinquefasciatus*. Our hypothesis was that in an insecticide treated environment we would observe a greater than additive interaction between resistance loci, whether present as homozygotes or heterozygotes. This hypothesis was tested by conducting bioassays on one insecticide susceptible strain, two pyrethroid resistant strains (with known resistance mechanisms) and crosses from these strains. The observed resistance levels were tested against null models to detect interactions.

Materials and methods

Mosquito strains

Three strains of *Cx. p. quinquefasciatus* Say were used. SLAB is a standard susceptible strain (Georghiou *et al.*, 1966). JPAL is a strain originating from Saudi Arabia (Amin & Hemingway, 1989) with high levels of resistance to phenoxybenzyl pyrethroids lacking an alpha substituent (Weerasinghe *et al.*, 2001). This high level of

resistance is due to *kdr* and cytochrome P450 monooxygenase-mediated detoxification (Kasai *et al.*, 1998). Metabolism studies showed that NADPH-dependent metabolism of permethrin to 4′-OH permethrin was greater in JPAL compared to a susceptible strain (Kasai *et al.*, 1998). The P450 responsible for this resistance has not been identified. Starting with JPAL and SLAB, standard backcrossing methods (Berticat *et al.*, 2002) were used to produce ISOP450, a 1300-fold permethrin resistant strain highly related to SLAB having the JPAL P450 resistance mechanism, but lacking *kdr* (Hardstone *et al.*, 2007). Permethrin resistance in ISOP450 is monofactorially inherited, incompletely dominant ($D = +0.3$) (Stone, 1968), and autosomally linked. It is expressed in the larvae, but not in adults (Hardstone *et al.*, 2007).

All mosquito colonies were reared by standard methods. Larvae were reared with ample developmental space in plastic trays containing 2 L distilled water for every 400 larvae. Larvae were provided abundant food consisting of a 1 : 2 : 1 mixture of ground TetraFin® goldfish flakes, rabbit pellets (The Hartz Mountain Corporation, Secausus, NJ, USA) and liver powder (MP Biomedicals, Aurora, OH, USA) in distilled water. Adults were provided a 20% sugar solution *ad libitum*, and provided a chicken as a blood source for 30 min two times per week (Cornell University Animal Use Protocol # 01–56). All life stages were maintained at 27 ± 1 °C, 80% RH, and photoperiod of 14 h : 10 h (L : D) including 2 h of simulated dawn and 2 h simulated dusk.

Bioassays

Resistance levels to permethrin (50 : 50, *cis* : *trans*; Chem Service, Westchester, PA, USA) for the parental strains and progeny from the crosses were determined by larval bioassay. All bioassays were performed over a 6-month period from January 2007 to June 2007. To determine the resistance conferred by the P450 detoxification mechanism as well as *kdr* individually, insecticide solutions were prepared with either acetone or in acetone containing piperonyl butoxide (PBO; Sigma-Aldrich, Inc., St Louis, MO, USA). Piperonyl butoxide is a P450 monooxygenase inhibitor, and treatment with this chemical plus the insecticide allows for detection and measurements of non-P450 mediated resistance mechanisms (i.e. *kdr*). It was previously determined that the maximum sublethal concentration of PBO was 1 mg mL^{-1} (Hardstone *et al.*, 2007). For each bioassay, 20 fourth instar larvae were placed in a 4-oz. wax coated paper cup (Sweetheart Cup Co., Owings Mills, MD, USA) with 99 mL of distilled water and 1 mL of insecticide solution. Control cups contained 99 mL of distilled water with 1 mL of acetone or 1 mL of PBO in acetone solution. All tests were run at 25 °C. Each bioassay consisted of at least 12 replicates per concentration and at least three (usually 4–6) concentrations of insecticide were tested that gave greater than 0% and less than 100% kill. Mortality was assessed after 24 h. Larvae were considered dead if they failed to move or resurface after being probed. Bioassay results were pooled and analysed by standard probit analysis (Finney, 1971), as adapted to personal computer use (Raymond, 1985), using Abbott's correction (Abbott, 1925) for control mortality.

To obtain mosquitoes with various combinations of genotypes, crosses were set up between existing strains. Individual pupae from each strain were kept in separate tubes. Upon eclosion, the respective sex and strain were released into a cage to create the desired cross. The adults were released *en masse* with a ~3 : 1 female : male ratio, with at least 200 females used. The following crosses were created: SLAB ♀ × ISOP450 ♂, ISOP450 ♀ × SLAB ♂, SLAB ♀ × JPAL ♂ and JPAL ♀ × SLAB ♂. Bioassays (see above) of each parental strain along with the progeny of the crosses were conducted.

Analysis of interaction between *kdr* and P450 detoxification resistance mechanisms

To determine if and what type of interaction was occurring between the two resistance loci (*kdr* and P450 detoxification) we first determined the toxicity of permethrin and permethrin + PBO in all mosquito strains and cross progeny. The toxicity information was used to determine the resistance contribution of each locus and the observed resistance levels were compared to the values expected from the models.

We tested for interactions between *kdr* and P450 detoxification loci using a null additive mathematical model. For this model, RR_1 and RR_2 are the resistance ratios conferred by the *kdr* and P450 detoxification mechanism, respectively, and RR_{12} is the resistance ratio of the two loci in combination. The additive model gives $RR_{12} = RR_1 + RR_2 - 1$ and we calculated the expected resistance conferred for each of the genotype combinations using the resistances measured for the individual mechanisms (Raymond *et al.*, 1989; Bohannan *et al.*, 1999). Additionally, interactions between the independent resistance loci were examined in models where the total two-locus genotypic contribution was plotted in relation to the phenotypic values (resistance ratios) on a linear scale and by logarithmically transforming the phenotype values.

Results

When the observed resistance ratios of each resistance loci were subjected to the three models to test for interactions (additive mathematical model, linear plot, and logarithmic plot), multiplicative interactions were found for all tested genotype combinations.

The levels of permethrin and permethrin + PBO toxicity in larvae of the parental strain and the progeny of the crosses are listed in Tables 1 and 2 respectively. When treated with permethrin, all reciprocal cross (SLAB

Table 1 Toxicity of permethrin to parental mosquito strains and F_1 progeny of crosses.

Strain (cross: female × male)	LC_{50}* (95% CI)	Slope (SD)	*n*†
SLAB‡	0.0017 (0.0015–0.0019)	4.5 (0.6)	320
JPAL‡	49 (45–54)	4.6 (0.4)	480
ISOP450‡	2.1 (1.9–2.5)	1.8 (0.1)	860
SLAB × JPAL	1.3 (1.1–1.6)	2.1 (0.2)	480
SLAB × ISOP450	0.16 (0.15–0.17)	2.6 (0.1)	1440
ISOP450 × SLAB	0.17 (0.15–0.19)	2.1 (0.1)	1800
ISOP450 × JPAL	11 (8.4–13)	2.3 (0.3)	1440
JPAL × ISOP450	16 (10–25)	3.4 (1.2)	1120

*Lethal concentration to kill 50% of the population in μg mL^{-1}.
†Total number of treated fourth instar larvae.
‡From Hardstone *et al.*, 2007.

Table 2 Toxicity of permethrin + PBO (a P450 inhibitor) to parental mosquito strains and F_1 progeny of crosses.

Strain (cross: female × male)	LC_{50}* (95% CI)	Slope (SD)	*n*†
SLAB‡	0.0011 (0.00090–0.0014)	7.7 (2.7)	480
JPAL‡	0.077 (0.049–0.12)	3.5 (1.4)	240
ISOP450‡	0.0059 (0.0053–0.0065)	3.0 (0.20)	960
SLAB × JPAL	0.011 (0.0094–0.012)	5.0 (0.54)	300
ISOP450 × JPAL	0.053 (0.048–0.058)	2.7 (0.2)	1310
JPAL × ISOP450	0.026 (0.020–0.034)	2.6 (0.4)	1540

*Lethal concentration to kill 50% of the population in μg mL^{-1}.
†Total number of treated fourth instar larvae.
‡From Hardstone *et al.*, 2007.

♀ × ISOP450 ♂ vs. ISOP450 ♀ × SLAB ♂ and JPAL ♀ × ISOP450 ♂ vs. ISOP450 ♀ × JPAL ♂) LC_{50} (concentration lethal to 50% of the population) values were not significantly different based on overlap of 95% confidence intervals. When larvae were treated with permethrin + PBO, the reciprocal crosses of JPAL ♀ × ISOP450 ♂ vs. ISOP450 ♀ × JPAL ♂ were slightly, but statistically different. The biological significance of the differing LC_{50} values is unknown although variation of this magnitude has been found amongst near isogenic strains (Hardstone *et al.*, 2006) and *kdr* has previously been shown to not be sex-linked (Halliday & Georghiou, 1985).

When treated with permethrin + PBO, resistance in JPAL was reduced to 70-fold. Resistance in ISOP450 treated with permethrin + PBO was 5.4-fold vs. 1300-fold when treated with permethrin only. This indicates that the major mechanism for permethrin resistance in the JPAL and ISOP450 strains was highly PBO suppressible (i.e. P450-mediated detoxification). These results are expected as previous studies showed JPAL contains both P450 detoxification and *kdr*, whereas ISOP450 contains only the P450 mechanism (Hardstone *et al.*, 2007). PBO treatment was able to suppress > 99.5% of the resistance in the ISOP450 strain. Therefore, the deduced relative contribution of *kdr* (Table 3) obtained from the bioassay of JPAL treated with permethrin + PBO is quite accurate, but is slightly inflated as there is a minor contribution of unsuppressed P450 detoxification to the resistance level. This is consistent with the 70-fold resistance to permethrin + PBO in JPAL being higher than the 34-fold resistance to permethrin previously reported for a *kdr* strain of *Cx. p. quinquefasciatus* (Scott *et al.*, 1986). The small amount of P450 detoxification that is not inhibited by PBO is also seen when resistance ratios of *kdr* heterozygotes are compared (Table 3).

The permethrin resistance conferred by the homozygous P450 mechanism (1300-fold) was greater than for homozygous *kdr* (70-fold). For both mechanisms, the heterozygotes conferred less resistance than the homozygotes. The P450 heterozygotes gave 94- to 100-fold resistance, and the *kdr* heterozygotes gave 10- to 48-fold permethrin resistance. Inability to completely inhibit the P450 mechanism with PBO likely contributes to the variation seen for the *kdr* heterozygotes. It is also possible that the variation seen for the *kdr* genotypes could be due to any differences present in the genetic backgrounds among the crosses.

When resistance levels were examined using the additive mathematical model, all observed resistance

Table 3 Resistance contributions of *kdr* and P450 detoxification in permethrin resistance.

Genotype			Resulting phenotype			
kdr	P450	Treatment	*kdr*	P450	RR*	Strain
S′/S′	R/R	Permethrin	S′/S′	R/R	1300	ISOP450
S′/S′	R/S	Permethrin	S′/S′	R/S	94	SLAB × ISOP450
S′/S′	R/S	Permethrin	S′/S′	R/S	100	ISOP450 × SLAB
R′/R′	R/R	Permethrin + PBO	R′/R′	S/S†	70	JPAL
R′/S′	R/S	Permethrin + PBO	R′/S′	S/S†	10	SLAB × JPAL
R′/S′	R/R	Permethrin + PBO	R′/S′	S/S†	48	ISOP450 × JPAL
R′/S′	R/R	Permethrin + PBO	R′/S′	S/S†	24	JPAL × ISOP450
S′/S′	R/R	Permethrin + PBO	S′/S′	S/S†	5.4	ISOP450

R/R, resistant homozygote; R/S, heterozygote; S/S, susceptible homozygote.
*RR (resistance ratio) = LC_{50} resistant strain/LC_{50} susceptible (SLAB) strain.
†Resistance mechanism largely suppressed by PBO.

Table 4 Observed permethrin resistance compared to the expected resistance under the additive mathematical model.

Genotype		Expected RR† additive mathematical			Observed
kdr	P450	model*	Observed RR†	Strain used for observed RR†	interaction
R′/R′	R/R	1300 + 70 − 1 = 1369	29 000	JPAL	Nonadditive
R′/S′	R/R	1300 + (10 to 48) − 1 = 1309 to 1347	6200 and 9300	ISOP450 × JPAL and JPAL × ISOP450	Nonadditive
R′/S′	R/S	(94 to 100) + (10 to 48) − 1 = 103 to 147	770	SLAB × JPAL	Nonadditive

*Additive mathematical interaction model: $RR_{12} = RR_1 + RR_2 - 1$ (Raymond *et al.*, 1989; Bohannan *et al.*, 1999). For mechanism genotypes with multiple RRs (see Table 3), RR range using the multiple expected values are shown for a comprehensive analysis of expected values.
†RR (resistance ratio) = LC_{50} resistant strain/LC_{50} susceptible strain.

ratios were greater than expected for additivity (Table 4). As some genotypes of the two mechanisms had multiple resistance ratios, all are included in the analysis to obtain a complete and comprehensive picture of the expected range of interaction values present in this system. When homozygous for both characters, the observed resistance was 29 000-fold, whereas the additive mathematical model predicted a resistance of 1369-fold. When homozygous for P450 detoxification and heterozygous for *kdr*, the observed relative resistances were 6200 and 9300-fold, whereas the additive mathematical model predicted resistance levels ranging from 1309 to 1347-fold. Finally, when both loci were heterozygous the observed resistance was 770-fold, but under the additive mathematical model the resistance levels were predicted to be from 103 to 147-fold. We were unable to obtain or isolate a strain that was heterozygous for the P450 character and homozygous for *kdr*, so we could not determine if the P450 heterozygote and *kdr* homozygote combination departed from additivity.

The plot models of phenotype values in relation to the two-locus genotype showed that on the linear scale the lines were not parallel (Fig. S1), however, when values were logarithmically transformed they were parallel (Fig. S2). There are two schools of thought for such results. One that considers epistasis as deviations from additivity on the linear scale, whereas the other considers epistasis as deviations from additivity on a log scale (Holland, 2001; Wade *et al.*, 2001; Cordell, 2002). Thus, our results could be variously classified. However, our results could be uniformly labelled as having multiplicative interactions.

Discussion

In mosquitoes, permethrin resistance is wide spread and conferred by two major mechanisms, *kdr* and P450 detoxification. Interactions between resistance loci (whether additive or nonadditive) are important to identify as these phenomena may contribute to high levels of resistance in the field, and understanding how mechanisms interact is important for more effective and targeted control strategies. We demonstrated that for *Cx. p. quinquefasciatus* there are multiplicative interactions between *kdr* and P450-mediated resistance mechanisms, whether the resistance alleles are homozygous or heterozygous.

The general trends reported in the literature for the interaction of insecticide resistance mechanisms are almost exclusively based on studies conducted on adult house flies (with a variety of resistance mechanisms) by topical application. When both resistance loci are homozygous, greater than additive interactions are generally observed (Hoyer & Plapp, 1966, 1968; Sawicki, 1970, 1973; Georghiou, 1971; Scott & Georghiou, 1986; Denholm *et al.*, 1992; Liu & Scott, 1995; Liu & Yue, 2001). When both resistance loci are heterozygous, additivity is generally observed (Georghiou, 1969; Sawicki, 1970; Scott *et al.*, 1984; Takada *et al.*, 1988; Lee & Shono, 1996; Park *et al.*, 1996; Zhang *et al.*, 1997; Shono *et al.*, 2002, 2004). However, there are exceptions (Tsukamoto, 1964; Sawicki & Farnham, 1967; Hoyer & Plapp, 1968; Georghiou, 1971).

Studies from the insecticide resistance literature, along with literature from other organisms, which examine interactions between independent loci, have shown that all three types of joint action exist. Our data show that there was significance for multiplicative interactions between *kdr* and P450 detoxification in all possible genotype combinations tested. Results from this study follow the general trend found when both insecticide resistance mechanisms are in the homozygous state, but does not follow the trend found in the house flies for interactions of heterozygous resistance loci.

The single previous study conducted on the interaction between mechanisms of resistance (insensitive acetylcholinesterase, B1 esterase and an unidentified mechanism) in mosquitoes (Raymond *et al.*, 1989) found that when all loci were heterozygous there was epistasis. However, the epistasis was not strictly multiplicative (i.e. $RR_{123} < RR_1 \times RR_2 \times RR_3$). Based on a pharmacokinetic model, they proposed the best fit to their results was a mixed interaction where resistance was equal to the unknown mechanism resistance ratio times the sum of the altered acetylcholinesterase and B1 esterase resistance ratios [$RR_{123} \sim RR_1 \times (RR_2 + RR_3)$].

The only other study that extensively examined combinations of resistance mechanism genotypes was Sawicki (1970) who investigated the interaction of *pen* and an unknown resistance gene (called gene *a*) in house flies. *Pen* alone provided 1.4-fold resistance to chlorthion-ethyl and 2.5-fold resistance to diazinon. Gene *a* alone provided 114-fold resistance to chlorthion-ethyl and 12.5-fold resistance to diazinon (Sawicki, 1970). When both mechanisms were in the homozygous state, there was a greater than additive interaction. Also, when either *pen* or gene *a* was homozygous with the other mechanism in the heterozygous condition, there was a greater than additive response. However, when both mechanisms were in the heterozygous state there was additivity (Sawicki, 1970).

In contrast to the studies on house flies, we found all tested combinations of *kdr* and P450 detoxification resulted in multiplicative interactions. In an evolutionary context, it is possible that both resistance loci will be maintained in the mosquito population if the fitness costs of both resistance alleles (in the absence of insecticides) are low. Future studies will be needed to determine the fitness cost/benefit of the *kdr* and P450 detoxification resistance loci in the absence of insecticide. By determining the presence (and direction) or absence of interactions occurring between resistance loci, we may be able to better predict the evolutionary trajectory of resistance.

Overall, interactions between independent loci, including those of insecticide resistance mechanisms, are complex. Many variables, such as the type of resistance mechanisms present, the fitness costs and benefits associated with the mechanisms, the insecticide compound investigated, the insect species and the definition of epistasis used, complicate the ability to formulate general interaction trends. It is therefore important and necessary to analyse each system individually and to consider the interactions between all possible genotypes as well as how the fitness costs/benefits of the mechanisms can manipulate the observed interactions. Although not directly investigated in this study, empirical observations of the strains used suggested that they were similar in their 'fitness' (i.e. produced ample offspring and were easily maintained). This suggests that in the absence of insecticides these resistance alleles do not necessarily exert a strong fitness disadvantage. However, future allele competition studies are needed to investigate this trend.

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Supporting information

Additional supporting information may be found in the online version of this article:

Figure S1 Plot on the linear scale of phenotypic values (resistance ratios) in relation to the total two-locus genotypic contribution of *kdr* and P450-detoxification.

Figure S2 Plot of logarithmically-transformed phenotypic values (resistance ratios) in relation to the total two-locus genotypic contribution of *kdr* and P450-detoxification.

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Supplementary Material

Figure S1 Plot on the linear scale of phenotypic values (resistance ratios) in relation to the total two-locus genotypic contribution of *kdr* and P450-detoxification.

Figure S2 Plot of logarithmically-transformed phenotypic values (resistance ratios) in relation to the total two-locus genotypic contribution of *kdr* and P450-detoxification.

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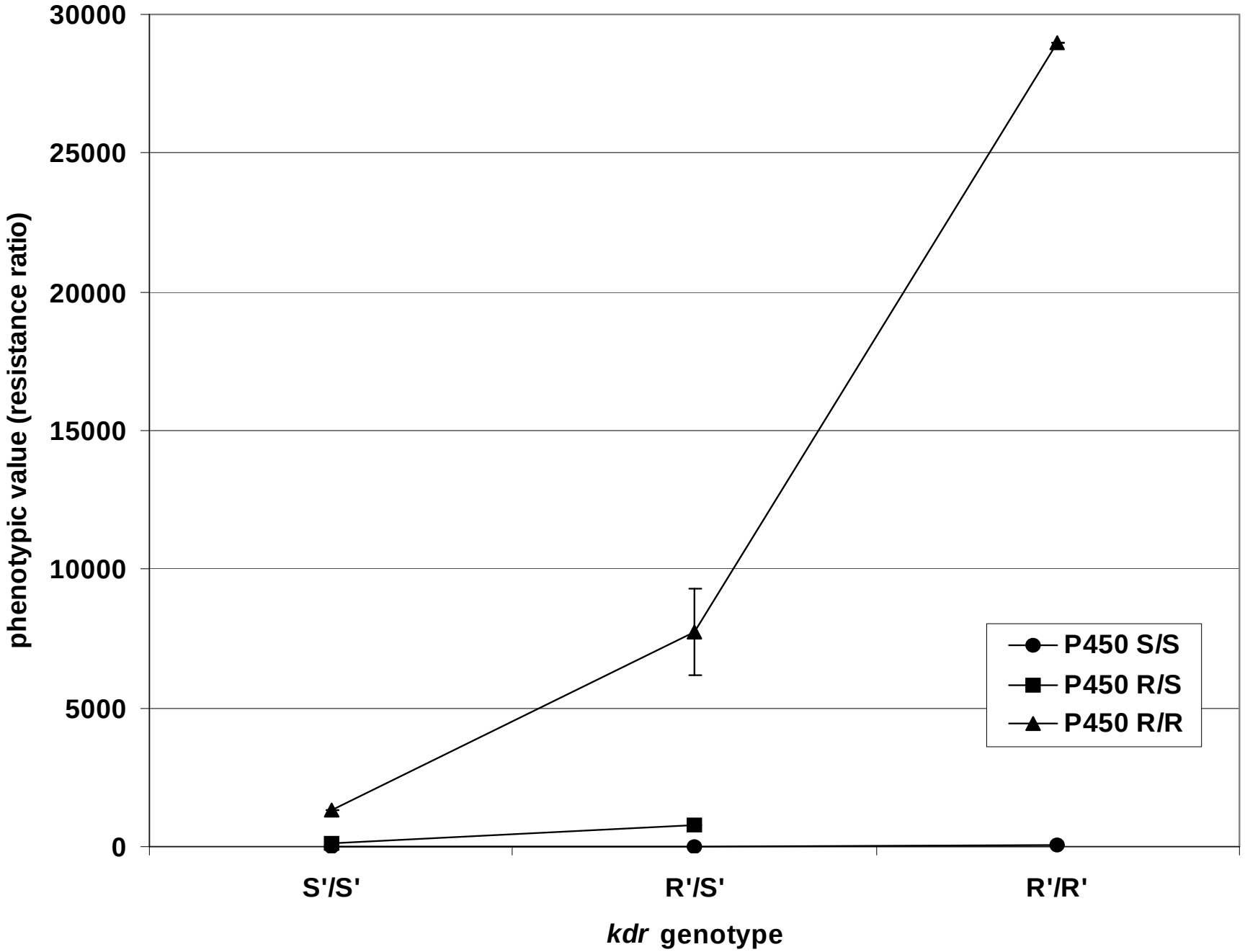
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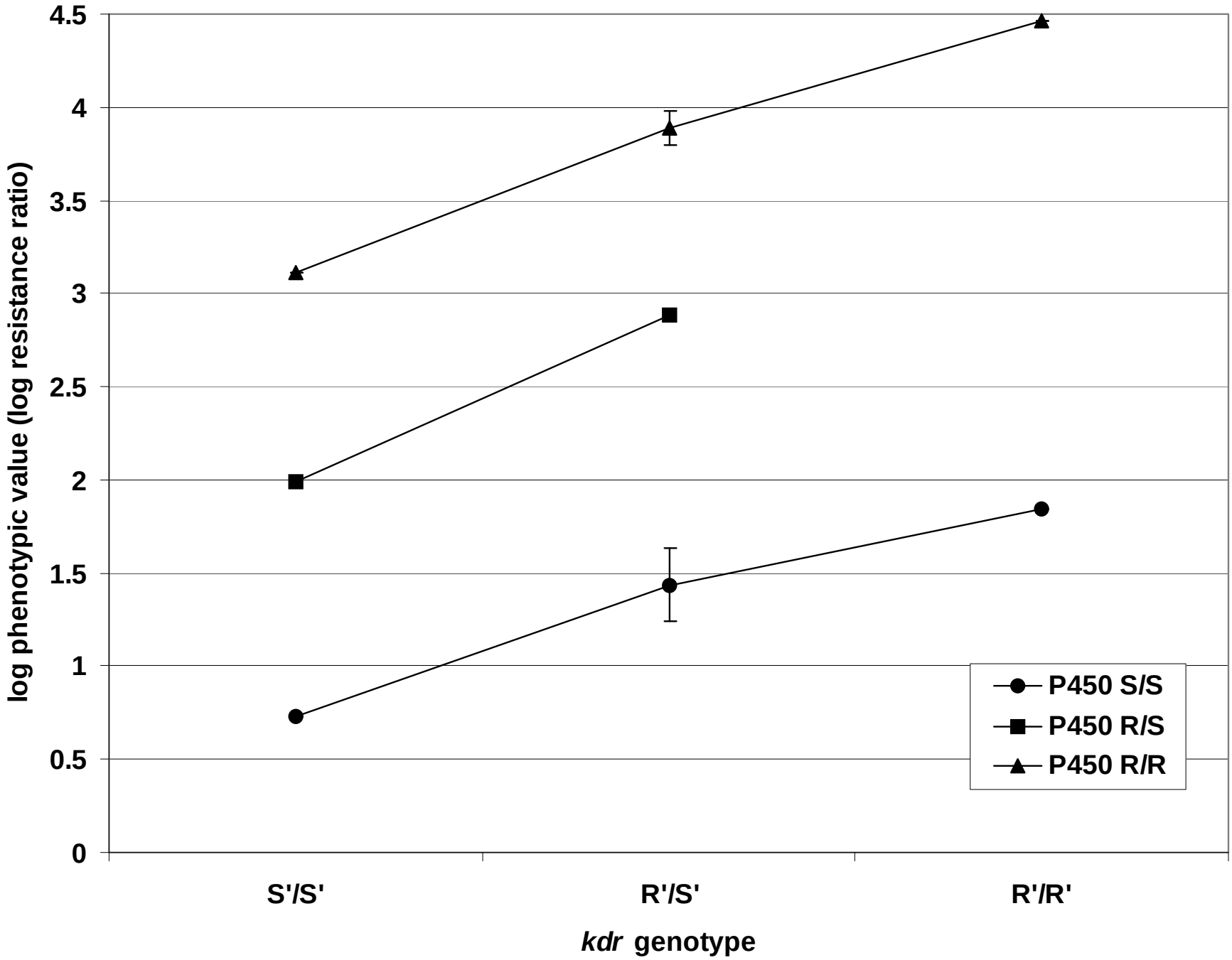
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