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Relative developmental and reproductive fitness associated with F1534C homozygous knockdown resistant gene in *Aedes aegypti* from Thailand

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Abstract. The effect of permethrin resistance, conferred by a homozygous mutation (F1534C) in the voltage-gated sodium channel protein, upon the reproductive fitness of *Aedes aegypti* (PMD-R strain) from Thailand was evaluated by comparing with a pyrethroid-susceptible subcolony (PMD strain). The parameters evaluated included larval development time, pupation success, adult emergence, egg production and hatchability, mating ability, female wing length and adult longevity. Larval development times were similar with very low mortality of larvae, pupae and emerging adults among either strain. However, PMD produced significantly fewer females than PMD-R. The mean numbers of eggs laid by PMD (54.2±15.9) and PMD-R (54.6±14.5) strains were not significantly different but the hatchability of PMD eggs (53.7%) was lower than PMD-R eggs (71.2%). The mean wing length of PMD females (2.85±0.15 mm) was longer than PMD-R females (2.74±0.09 mm). The insemination rates for both strains were 100%. The longevity of both strains was mostly not significantly different, over 90% of both sexes surviving at day 30. Our results suggest that the presence of the homozygous F1534C mutation does not lead to fitness reductions. This is in accordance with the high frequency of this allele found among wild populations of *Ae. aegypti* in many countries. These results also suggest that the removal of pyrethroid insecticide selection pressure may not lead to a regression of 1534C alleles in pyrethroid resistant *Ae. aegypti*.

INTRODUCTION

Aedes aegypti is the major vector of viral diseases including dengue and dengue hemorrhagic fever, and chikungunya which are serious public health problems in Thailand as well as many countries in tropical and subtropical areas. Because a dengue vaccine and specific treatment are not available, control of transmission is basically based on management of breeding places, or the application of larvicidal (e.g. temephos sand granules) and adulticidal chemicals (e.g. fogging and ultra-low-volume sprays). Many insecticides including Dichlorodiphenyltrichloroethane (DDT), the organophosphates (e.g., malathion, fenitrothion, and temephos), and carbamate (e.g., propoxur) were heavily used for mosquito control for over 50 years in Thailand before being replaced (except temephos) by pyrethroids in the early 1990s (Chareonviriyaphap *et al.*, 1999). The adverse effect of the heavy and long-term use of insecticides is resistance of *Ae. aegypti* worldwide. In Thailand, resistance to DDT was first reported in *Ae. aegypti* in the mid 1960s (Neely, 1966). At present, it is known to be resistant to several insecticides, particularly pyrethroids (i.e., permethrin and deltamethrin), organophosphate compounds (i.e., temephos and fenitrothion), and carbamate

compounds (i.e., propoxur) (Somboon *et al.*, 2003; Ponlawat *et al.*, 2005; Jirakanjanakit *et al.*, 2007). This problem has severely hampered the control of vectors by insecticides.

There are two broad classes of resistance mechanisms that play an important role in mosquito resistance to insecticides: target site insensitivity and metabolic enzyme-based resistance (Hemingway & Ranson, 2000). Target site insensitivity to pyrethroids and DDT in mosquitoes and other insects is associated with single or multiple mutations, commonly referred to as knockdown resistance (*kdr*). These mutations modify the voltage-gated sodium channel protein, making it less susceptible to the binding of pyrethroids and DDT (Soderlund & Knipple, 2003). Metabolic enzyme-based resistance is principally associated with three enzyme groups: cytochrome P450 monooxygenases (P450s), esterases, and glutathione-S-transferases, depending on the insect species/strain and the insecticide (Hemingway & Ranson, 2000).

The heavy use of insecticides in mosquito control programmes may cause a dramatic increase in the frequency of resistant alleles (Garcia *et al.*, 2009; Martins *et al.*, 2009). In the absence of insecticide pressure, however, reduced fitness of resistant insects is frequently reported, resulting in a reproductive disadvantage, which would probably decrease resistant allele frequencies in the field over time (Lenormand *et al.*, 1999). Several studies have shown that resistance to insecticides reduces, to some degree, the reproductive fitness of *Ae. aegypti* (Mebrahtu *et al.*, 1997; Kumar *et al.*, 2009; Belinato *et al.*, 2012; Martins *et al.*, 2010) as well as some *Culex* and *Anopheles* mosquito species (e.g. Amin & White, 1984; Rowland, 1991; Wang *et al.*, 1998; Hardstone *et al.*, 2009; Kumar & Pillai, 2011). The fitness reductions observed in insecticide-selected strains is generally considered due to pleiotropy of the resistant alleles or to a hitch-hiking effect (Smith & Haigh, 1974). By contrast, Okoye *et al.* (2007) reported that pyrethroid resistance in southern African *Anopheles funestus* does not incur any loss of fitness under laboratory conditions. In addition, other insect species, including boll weevils, houseflies and cockroaches, do not show differences in fitness between resistant and susceptible strains in the absence of insecticide treatment (Varzandeh *et al.*,, 1954; Perkins & Grayson, 1961; Thomas & Brazzel, 1961; Roush & Hoy, 1981). These studies have suggested that resistance and fitness may evolve independently (Heather, 1982) and differences in insecticide resistant mechanisms or alleles may affect fitness positively or negatively (Berticat *et al.*, 2008; Rivero *et al.*, 2010). It is, however, difficult to associate fitness disadvantages specifically with resistance in field populations. Estimates of relative reproductive and survival rates of resistant and susceptible genotypes obtained from laboratory studies are useful when considering the influence of resistance alone on biological fitness. As most previous studies concerning reproductive fitness of resistant *Ae. aegypti* are associated with metabolic resistance, little is known about the effect of knockdown resistance genes in regards to fitness and survival. The aim of this study was to determine whether pyrethroid resistance in *Ae. aegypti* conferred mainly by knockdown resistant mechanism affects certain fitness components when compared to the susceptible sub-colony. Understanding these aspects is essential for improving resistance monitoring, detection and management in vector control programmes in Thailand.

MATERIALS AND METHODS

Mosquito strains

Two laboratory strains of *Ae. aegypti*, PMD and PMD-R, were used in this study. Both originated from Ban Pang Mai Daeng, Mae Tang District, Chiang Mai Province, Thailand (Prapanthadara *et al.*, 2002). They have been maintained over 10 years in our insectary at 25±2ºC, 70-80% RH and 14 h illumination. The PMD-R strain is resistant to both DDT and permethrin while PMD is resistant to DDT but susceptible to permethrin. Permethrin resistance in the PMD-R strain is mainly due to the homozygous mutation in codon F1552

of the *kdr* gene of *Ae. aegypti* (equivalent to F1534 in the house fly *Vssc1* sequence) resulting in the replacement of phenylalanine with cysteine in segment six domain III of the voltage-gated sodium channel protein (Somwang *et al.*, 2011; Yanola *et al.*, 2010). This mutation (F1534C) is common throughout Thailand (~0.8 allele frequency, Yanola *et al.*, 2011) and is widespread in Vietnam (up to ~0.87) (Kawada *et al.*, 2009) and Grand Cayman (0.68) (Harris *et al.*, 2010). In the PMD strain, no *kdr* mutation has been observed in domains II or III of the voltage-gated sodium channel protein (Yanola *et al.*, 2010, 2011). Our previous biochemical characterization revealed that the levels of total P450s, DDTase, esterase and glutathione-S-transferase (GST) were similar in both strains. However, there was a tenfold increase in DDTase activity and a fourfold increase in P450 activity compared to the susceptible Rockefeller strain, whereas the esterase and glutathione-S-transferase (GST) activities were only slightly increased (Prapanthadara *et al.*, 2002; Somwang *et al.*, 2011). The PMD-R adult mosquitoes have been maintained under regular insecticide pressure (0.75% permethrin) using standard WHO kits (WHO, 1975).

Exp. 1: Larval and pupal development and adult emergence

Stocks of previously dried eggs were submerged in distilled water for 1-2 days and newly hatched first instar larvae were randomly collected. For each strain, triplicates of one hundred first instar larvae were reared in 0.5 liter cups containing 480 ml of distilled water. On the first day, 0.15 g of ground fish food was offered and added every other day until the end of the experiment. The first day of pupation, mean pupation time, pupation rate and emergence rate were recorded. Newly emerged male and female mosquitoes were placed in 30 cm^3 cages covered with moist towel and used for Exp. 2.

Exp. 2: Insemination, fecundity, hatchability of eggs, size of females

Adult mosquitoes from Exp. 1 were kept in the cage for a week. A blood meal was then offered and the fully engorged females were randomly aspirated and kept individually in 50 ml cups lined with filter paper. They were provided with a 10% sugar solution. On day 5 post feeding a small amount of distilled water was put into the cup for oviposition. The resulting eggs were counted and air dried for a week. To determine the hatchability of the eggs, they were submerged in distilled water for 3 days and hatched larvae were counted. After oviposition, the females were dissected for spermathecae to determine the presence of spermatozoa. The degree of insemination was scored as 4+, 3+, 2+, 1+ or 0. A score of 4+ indicates the presence of spermatozoa filling all three lobes of the spermathecae, 3+ indicates the presence of spermatozoa in 2 lobes and only scanty spermatozoa in the other, 2+ signifies the presence of spermatozoa filling one lobe and scanty in the others, 1+ denotes the presence of only scanty spermatozoa in one or more lobes, and a score of 0 was given if no insemination occurred.

After the dissection of spermathecae, female mosquito size was determined by measuring the length of the right wing from the axillary incision to the wing tip excluding fringe. The measurement was performed under a stereomicroscope with calibrated ocular micrometer.

Exp. 3: Longevity

The longevity of adult mosquitoes fed on sugar solution was determined. Triplicates consisting of one hundred pairs of one day old male and female mosquitoes reared from larvae as described in Exp. 1 were placed in 30 cm^3 cages covered with moist towel. Cage floors were lined with white paper so that dead mosquitoes were easily seen. They were provided with a 10% sugar solution soaked onto cotton wool which was changed every other day. Dead mosquitoes were counted and removed daily. Similarly, the longevity of adult mosquitoes under starvation conditions was investigated by rearing under similar conditions but providing only water instead of a sugar solution.

Data were analyzed by using SPSS version 12.0.1. Student *t*-test was employed for comparing mean values of larval and

pupal development, adult emergence, egg production, hatchability, longevity and wing length. Chi-square test was performed for sex ratio and insemination rate.

RESULTS

Exp. 1: Larval and pupal development and adult emergence

Developmental times from first instar larvae to pupae were similar for both strains (Table 1). Pupation began on the tenth day, with mean pupation times (L1 to 50% pupation) being 14.3±0.9 and 15.2±0.6 days for PMD and PMD-R strains respectively. A total of 282 and 293 pupae were obtained from the PMD and PMD-R respectively. Percent pupation success and adult emergence success of the two strains were not significantly different. The observed sex ratios (M:F) of emerging adults of PMD was 165:102 (P = 0.007) and of PMD-R 126:159 (P =0.178), indicating that PMD produced 17.6% fewer females than PMD-R.

Exp. 2: Insemination, fecundity, hatchability of eggs, female size

There was no significant difference between the PMD and PMD-R strains regarding mean numbers of eggs laid (Table 2). Dissection of spermathecae revealed that all females from both strains were inseminated. The quantity of spermatozoa in PMD showed 11.4% of 4+, 84.1% of 3+, and 4.5% of 2+, and in PMD-R 5.4% of 4+, 91.9% of 3+, and 2.7% of 2+, which were not significantly different (X^2=1.23, d.f. =2, P >0.5). However, the mean number of hatching larvae per PMD female (average 53.7%) was significantly lower than PMD-R (average 71.2%). In addition, the mean wing length of PMD females was significantly longer than PMD-R females.

Exp. 3: Longevity

Provided with a 10% sugar solution, the average survival rates of males and females from both strains determined at 30 and 45 days post-emergence were not significantly different (Table 3). However, at day 60 when the experiment was concluded, more PMD-R

Table 1. Mean (±SD) developmental time (days), pupation success, adult emergence success and sex ratio of *Ae. aegypti* permethrin susceptible (PMD) and resistant (PMD-R) strains. Ranges (min–max) are given in parentheses

	PMD	PMD-R	P
L1 to first day of pupation (days)	10.7±0.6 (10-11)	10.0±0.0 (10)	0.374
L1 to 50% pupation (days)	14.3±0.9 (13.6-15.3)	15.2±0.6 (14.5-15.7)	0.249
L1 to pupation success (%)	97.2±1.5 (96.9-98.9)	98.8±1.1 (97.9-100)	0.349
Pupae to emerging adults (%)	98.2±0.6 (97.9-99.0)	97.9±1.1 (96.8-98.9)	0.408
Sex ratio (Male: Female)	165:102		0.007
Sex ratio (Male: Female)		126:159	0.178

Table 2. Mean (±SD) egg production, hatchability and wing length of the *Ae. aegypti* permethrin susceptible (PMD) and resistant (PMD-R) strains. Ranges (min–max) are given in parentheses

	PMD (n=43)	PMD-R (n=37)	P
number of eggs per female	54.2±15.9 (15-82)	54.6±14.5(12-78)	0.910
number of hatching larvae per female	29.1±17.1 (0-59)	38.9±12.6 (7-66)	0.005
wing length of females (mm)	2.85±0.15 (2.53-3.13)	2.74±0.09 (2.53-2.93)	<0.001

Table 3. Longevity of the *Ae. aegypti* permethrin susceptible (PMD) and resistant (PMD-R) strains provided with 10% sugar solution and water. Mean (±SD) and ranges (min–max) are given in parentheses

Day	% survival		P
	PMD	PMD-R	
With sugar			
30: Male	91.0±3.6 (87-94)	95.0±2.6 (93-98)	0.217
Female	95.7±3.8 (93-100)	97.7±1.5 (96-99)	0.444
45: Male	78.7±6.4 (74-86)	89.3±2.5 (87-92)	0.055
Female	92.7±3.1 (90-96)	95.3±2.1 (93-97)	0.280
60: Male	66.3±9.1 (58-76)	81.0±4.0 (81-85)	0.063
Female	82.0±1.0 (81-83)	88.3±2.5 (86-91)	0.015
With water			
3: Male	99.0±1.0 (98-100)	99.3±0.58 (99-100)	0.643
Female	98.3±1.2 (97-99)	96.7±5.8 (90-100)	0.649
6: Male	25.0±16.1 (12-43)	33.7±14.6 (17-44)	0.527
Female	15.3±8.5 (9-25)	18.0±13.9 (9-34)	0.791
9: Male	11.7±9.5 (1-19)	2.0±1.7 (0-3)	0.216
Female	12.0±11.1 (2-24)	3.0±2.0 (1-5)	0.240

females survived. When only water was provided, no significant differences of the average survival rates determined at 3, 6 and 9 days post-emergence were observed. Few individual males and females of both strains died in the first 3 days. About a half of the males and females from both strains died on day 4 and none survived after 12 days.

DISCUSSION

This study compared the reproductive fitness between the permethrin susceptible PMD and resistant PMD-R strains of *Ae. aegypti* in laboratory conditions. Both strains share a close genetic background since they originated from the same area and are resistant to DDT with similar levels of the major metabolic detoxification enzymes (Prapanthadara *et al*, 2002; Somwang *et al.*, 2011). Unfortunately, *Ae. aegypti* populations in Thailand are widely resistant to DDT (Somboon *et al.*, 2003; Ponlawat *et al.*, 2005) and hence a DDT-susceptible strain was unavailable for comparison. Nevertheless, the main difference between the two strains is the presence of the F1534C homozygous mutation in the PMD-R strain which allowed us to determine the relative fitness cost associated with *kdr* gene.

Larval development, pupation success, adult emergence and mating ability were considered normal and not significantly different between both strains. These results suggest that the presence of F1534C homozygous mutation does not have a negative impact on these parameters. Significant differences were observed between the number of females produced (PMD < PMD-R), the mean wing length of females (PMD > PMD-R), the mean number of hatching larvae per female (PMD < PMD-R) and the female survival rate (PMD < PMD-R) at day 60. PMD-R females, as determined by the mean wing length, were smaller than PMD females (Table 2), suggesting that the *kdr* gene may also affect the body size of mosquitoes. Bourguet *et al.* (2004) reported that overproduced acetylcholinesterase *Culex pipiens* showed shorter wing length. Also, Harstone *et al.* (2009) revealed that *Culex quinquefasciatus* mosquitoes resistant to pyrethroid due to elevated P450

were smaller than those of the susceptible strain. Larger female mosquitoes are generally considered to produce more eggs (Clements, 1992), but this was not observed in the present study.

Compared with the Rockefeller strain and the susceptible group of *Ae. aegypti* reported in Martins *et al.* (2010), the mean numbers of eggs and the hatchability of PMD-R and PMD strains were, respectively, about 60-70% and 40-70% lower. Although a direct comparison with these strains cannot be fully made due to different laboratory conditions and genetic background, these results suggest a relatively low reproductive fitness in both PMD and PMD-R strains, with a lesser extent in the latter. Although the amount of ingested blood, which is directly related to the number of eggs deposited, was not investigated, it is likely that insecticide resistant females consumed lower amounts of blood (Li *et al.*, 2002; Belinato *et al.*, 2012; Martins *et al.*, 2010) or required a higher number of blood meals to lay eggs (Okoye *et al.*, 2007).

The reduced egg hatchability in PMD could not be explained due to the reduced mating ability (as reported in Belinato *et al.*, 2012) since the insemination rates and the quantity of spermatozoa in the spermathecae were similar in both strains. The egg hatchability of PMD was 17.5% lower than PMD-R (Table 2). This was almost equal to the difference between the reduced number of females produced by PMD compared to PMD-R (17.6%) (Table 1). We expect that a number of female eggs of PMD were not hatchable or their hatchability was delayed or required re-submerging to hatch, resulting in significantly fewer adult females produced by PMD in Exp. 1. The PMD strain has been maintained in our laboratory for many years without insecticide pressure but the level of DDT resistance was only slightly decreased (unpublished data). By contrast the PMD-R strain has been regularly exposed to permethrin. It is possible that the F1534C mutation is favoured in insecticide treated environments. Thus, the reduced egg hatchability and diverted sex ratio in PMD are likely associated with a fitness cost (Rivero *et al.*, 2010). Relative reductions in the number of eggs laid and hatchability have often been reported in insecticide-resistant *Ae. aegypti* compared against susceptible strains (e.g. Mebrahtu *et al.*, 1997; Kumar *et al.*, 2009; Belinato *et al.*, 2012; Martins *et al.*, 2012). In *An. funestus*, however, Okoye *et al.* (2007) reported that the mean numbers of laid eggs between the pyrethroid resistant and susceptible strains were not significantly different, but the mean number of larvae per female and the mean number of females produced were significantly higher in the resistant strain as found in the present study.

In the experimental conditions of this study, the longevity of PMD and PMD-R adults provided with the sugar solution was almost similar, except for PMD-R females that survived slightly longer at day 60, suggesting again a slightly relatively low fitness of PMD in longevity (Table 3). Hardstone *et al.* (2009) also reported that when provided with sugar *Cx. quinquefasciatus* females resistant to permethrin survived longer than susceptible ones. However, there was no significant difference when they were provided with water as observed in the present study. Over 66% and 82% of male and female mosquitoes, respectively, survived up to 60 days which was slightly longer than *Ae. aegypti* strains in other reports (e.g. Belinato *et al.*, 2012; Martins *et al.*, 2010). This may be explained in part because their mosquito materials were newly selected or introduced whereas our colonies were well-adapted to laboratory conditions for many years. Braks *et al.* (2006) also reported that the medium survival time of laboratory adapted *Ae. aegypti* females was 57.18 days. This high survival, however, cannot reflect the longevity of wild *Ae. aegypti* mosquitoes which usually live for a few weeks (Scott *et al.*, 1997). Therefore, the present study does not necessarily suggest that the F1534C mutation shows greater fitness on longevity than wild population, but suggests that the mutation does not have negative impact on longevity of *Ae. aegypti* adults. Belinato *et al.* (2012) also revealed that the longevity of temephos and deltamethrin resistant *Ae. aegypti* with the V1016I mutation was not significantly different from the Rockefeller strain.

In nature, although female *Ae. aegypti* fed predominantly on humans and seldom fed on plant sugar (Edman *et al.*, 1992; Scott *et al.*, 1993), addition of sugar solution in rearing increased longevity of blood-fed mosquitoes (Braks *et al.*, 2006). The tolerance to starvation, with deprivation of energy resources derived from the immature stages, was evaluated by providing PMD and PMD-R adults with only water. Almost all males and females survived for 3 days without sugar which is considered long enough for the males to mate and for the females to find a blood meal. Paris *et al.* (2011) found no difference in adult starvation tolerance in an *Ae. aegypti* strain selected with *Bacillus thuringiensis* var. *israelensis* toxins. Similarly, Hardstone *et al.* (2009) reported that the median longevity of susceptible and resistant *Cx. quinquefasciatus* mosquitoes with only water was 4 days. However, Agnew *et al.* (2004) reported greater vulnerability to starvation for three *Cx. quinquefasciatus* strains homozygous for the *ester*1, *ester*4 or *Ace-1*R allele, all of which are related to resistance to organophosphate insecticides.

Reductions in reproductive fitness, including the size of insecticide-resistant insects are generally explained due to pleiotropic effects (Rivero *et al.*, 2011), reducing the energy and nutrition available for other biological functions and generating energetic trade-offs between insecticide resistance and key life history traits. However, large variations in fitness or even the absence of fitness costs have been reported, depending on insect species, genetic background and resistance mechanisms. For example, Belinato *et al.* (2012) reported that Brazilian *Ae. aegypti* highly resistant to temephos exhibited decreases in blood meal acceptance, amount of ingested blood, egg production and frequency of inseminated females, whereas the strain with a lower temephos resistance level presented impairment in only blood meal acceptance and frequency of inseminated females. Similarly, Martins *et al.* (2010) revealed that *Ae. aegypti* obtained from wild populations in Brazil, when selected with deltamethrin in the laboratory, displayed numerous fitness costs among the resistant groups including delayed larval development, low pupation, longevity reduction and reduced number of laid eggs and hatchability. In a strain resistant only to temephos, only delayed larval development was observed.

Inbreeding depression can also reduce the fitness of mosquito colonies under laboratory conditions (Munstermann, 1994; Armbruster *et al.*, 2000), but this effect, if any, is considered small in PMD and PMD-R strains because they have been maintained in our laboratory for many years without serious deteriorative signs, e.g. high mortality of immature stages and reduced longevity of adults.

In conclusion, the overall parameters evaluated suggest that the presence of the F1534C homozygous mutation is unlikely to have a serious negative effect on reproductive fitness of mutant *Ae. aegypti* compared to non-mutant populations. This finding is in accordance with the widespread distribution of the F1534C mutation in southeast Asia (so far detected in Thailand, Vietnam, Myanmar and Cambodia) and Grand Cayman (Harris *et al.*, 2010) with high mutant allele frequencies (0.68-0.87) whereas the wild type, like the permethrin-susceptible PMD strain, is found in only <1% of the wild population of *Ae. aegypti* in Thailand (Yanola *et al.*, 2011). More recently in 2012, examination of *Ae. aegypti* larvae collected from several containers from Lahore, Pakistan, where pyrethroids have been heavily used for dengue control, revealed 100% (n = 23) were 1534C homozygous mutant (unpublished data). In addition, previous reports have revealed that another *kdr* mutation (V1016I) has spread rapidly among field populations of *Ae. aegypti* in Brazil and Mexico (Garcia *et al.*, 2009; Martins *et al.*, 2009). These studies suggest that current insecticide-based control programmes may not be able to reduce *kdr* alleles in wild populations. With the relaxation of insecticide selection pressure, however, Chang *et al.* (2012) demonstrated in the laboratory that the *kdr* allele frequencies (V1016G and D1763Y) in permethrin resistant *Ae. aegypti* declined to about 20% after 15 generations. Whether this reversal would occur for F1534C and other

point mutations in the laboratory or in the field requires further study.

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