SEASONAL AND SPATIAL VARIATION IN NATURAL DENSOVIRUS INFECTION IN *ANOPHELES MINIMUS S.L.* IN THAILAND

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Abstract. We report the first detection of a mosquito densovirus in anophelines, *An. minimus* species A and species C, and describe temporal and spatial variation in natural densovirus infection. A total of 814 (136 species A; 678 species C) adult mosquitos, obtained over a one year period from human biting catches at three locations in a village in western Thailand, were PCR tested for densovirus infection. Overall infection prevalence did not differ between species (15.4% species A; 14.5% species C). Infection prevalence showed significant seasonal variation. Some spatial heterogeneity in infection was also noted, with timing of peak infection prevalence varying between sites. PCR-screening of *An. minimus s.l.* larvae found an overall infection prevalence of 18.8%. Larval infection showed a significant positive association with rainfall recorded two months previous to larval collection. Infection in adult mosquitos showed a moderate relationship to environmental variables, but a significant negative correlation with larval infection in the previous month.

INTRODUCTION

Insect densoviruses are small autonomous non-enveloped DNA viruses that belong to the family Parvoviridae. They are characterized by the presence of inverted terminal repeat sequences and the separate encapsidation of complementary single-stranded DNA. Two subgroups insect densoviruses have been recognized on the basis of lepidopteran isolates (Tijssen and Arella, 1991). Mosquito densoviruses do not easily fit into the same classification scheme, and thus form a third subgroup. They have been described to date from *Aedes aegypti* (*A*DNV) (Afanasiev *et al*, 1991; Lebedeva *et al*, 1973) and from cell lines of *Ae. albopictus* (*Aa*PV) (Jousset *et al*, 1993), *Ae. pseudoscutellaris* (Gorziglia *et al*, 1980), *Toxorhynchites amboinensis* (*Ta*DNV) and *Haemogogus equinus* (*He*DNV) (O'Neill *et al*, 1995). Recently, another mosquito densovirus was described from both *Ae. aegypti* and *Ae. albopictus* in Thailand (*ATh*DNV) (Kittayapong *et al*, 1999).

Densoviruses have been shown to cause lethal disease in their arthropod hosts, while attempts to experimentally infect vertebrates with these viruses have failed (Jousset *et al*, 1993, Kawase *et al*, 1990). *A*DNV was noted to be pathogenic for *Aedes*, *Culex*, and *Culiseta* larvae when introduced into the water in which they were reared, although *Anopheles* larvae could only be infected by injection (Buchatsky, 1989). *Aa*PV was found to be highly lethal for *Ae. aegypti*, killing up to 95% of larvae after infection orally (Jousset *et al*, 1993; Barreau *et al*, 1996). Histopathological examination of infected larvae showed the virus to be polytrophic and mortality was attributed to destruction of fat body cells (Barreau *et al*, 1996). Over 80% of *Ae. albopictus* and 50% of *Ae. aegypti* larvae died after being orally infected with *ATh*DNV (Kittayapong *et al*, 1999).

Mosquito densoviruses are believed to be maintained in nature primarily by horizontal transmission, although transovarial and venereal transmission have also been recorded (Barreau *et al*, 1997; O'Neill *et al*, 1995, Kittayapong *et al*, 1999). These viruses are of particular interest for their potential use in both biological control and genetic manipulation of insect disease vectors. However, little is known about the ecology of densovirus infection in natural mosquito populations. Although it has been suggested that mosquito densoviruses may be widespread, systematic surveys of infection in mosquito species have not been undertaken. Furthermore, most experimental data on virus infectivity and pathogenicity have been gathered on *Aedes*. We report on the first detection of a mos-

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quito densovirus in anophelines, *Anopheles minimus* species A and species C, two of the major vectors of malaria in SE Asia. In addition, we describe the temporal and spatial heterogeneity of natural densovirus infection in this species complex.

MATERIALS AND METHODS

Study location

The study was conducted in Ban Phu Toei, a village of approximately 350 inhabitants in Sai Yok district, Kanchanaburi Province, western Thailand. Ban Pu Toei is located 30 km from the border with Myanmar, at 100 m above sea level, latitude 14.3ºN and longitude 98.5ºE. Annual rainfall is approximately 1.5 m, with distinct wet and dry seasons. The village rests in a small valley between forested hills which rise to elevations of approximately 400 m. Surrounding vegetation is primarily agricultural land of rice and maize, abandoned cropland that has been overgrown, or dense secondary forest. The region is highly malarious with both *Plasmodium falciparum* and *P. vivax* infections that are resistant to many anti-malarial drugs including chloroquine. The principal malaria vectors are the *An. minimus* and the *An. dirus* species complexes.

Mosquito specimens

a) Adult collections: Adult *An. minimus* species A and C were collected once monthly from January to December 1997. Mosquitos were captured from three fixed locations in the village, using human landing techniques: 1) indoor; 2) peridomestic, approximately 20 m from human dwellings; and 3) forest fringe, approximately 300 m from human dwellings. Collections were made on two consecutive nights by two persons at each site, between the hours of 18.00 hours to midnight.

Mosquitos were identified using morphological keys of Buei (1983), Peyton and Scanlon (1966) and Rattanarithikul and Panthusiri (1994). On the basis of the presence of humeral pale spots on their wings, *An. minimus* species C will be correctly identified in 95% of instances (Green *et al*, 1990; Sharpe, 1997). *An. minimus* species A, which generally lack humeral pale spots, will be correctly identified in 73% of instances. After identification, specimens were frozen at -20ºC until PCR assay for the densovirus genome. On average, 21 specimens from each collection location were PCR tested for each month.

b) Larval collections: *An. minimus s.l.* larvae were collected monthly from February through December 1997 from an additional four sites in Ban Phu Toei, each representing a different larval habitat. Site A was a shallow stream located 20 m from an occupied house. Site B was an intermittent swampy pool with little movement of water, and Site C was a slow-flowing shallow stream on the edge of a rice field. Both these sites were over 200 m from human dwellings. Site D was a clear pool on the edge of a fast-moving stream in the forest, approximately one kilometer from the village. Larvae were collected with a strainer, and kept in a plastic bag with water from the same habitat. They were identified using morphological keys (Harrison, 1980) and kept at -20ºC for later use. Attempts to identify larval *An. minimus* to the species level were not made, thus classification was as *sensu latu*.

Environmental data

Monthly climate data including rainfall, humidity and temperature were obtained for Kanchanaburi Province from the Meteorological Department in Bangkok. The station where the climate data were recorded is approximately 40 km from Ban Phu Toei village, thus provides a rough estimate of environmental conditions in the village for each month. The year was divided into three seasons, based on recorded rainfall and temperature values. Dry cool season months, from November through February, had less than five days of rain and mean temperatures less than 28ºC. The hot season, from March through June, were months with mean maximum temperatures over 37ºC. Rainy season months, from July through November, had more than five days of rain and mean temperatures less than 30ºC.

PCR amplification of viral genome

Mosquitos were PCR-screened for the densovirus genome using primers designed from the densovirus ORF3 (O'Neil *et al*, 1995). Crude DNA extractions were performed by homogenizing an individual adult or a larval mosquito in 100 µl of STE buffer, using the methods of O'Neill *et al* (1992). One microliter of the supernatant was used as DNA template in the PCR reaction. Densovirus-infected *Ae. aegypti* were used as a positive control. Negative controls were randomly included to check for contamination. Viral DNA was PCR amplified in 20 µl reaction volumes: 2 µl 10x buffer (Promega), 2 µl 25 mM $MgCl_2$, 0.5

µl dNTPs (10 mM each), 0.5 µl of each primer and 1 unit of *Taq* DNA polymerase (Promega). The PCR thermal profile was 95ºC 1 minute, 50ºC 1 minute and 72ºC 1 minute per cycle for 35 cycles. PCR products were run on a 1% agarose gel with a 1Kb ladder (Gibco) to determine the presence and size of amplified DNA. Samples that yielded products of the expected size (350 bp) were scored as positive.

Universal eukaryotic 12S rDNA primers, which should yield positive results from all insects, were used to check the quality of the DNA extraction on samples which tested negative for densovirus. Samples that were negative for 12S were excluded from the data. To confirm the presence of densovirus, some densovirus PCR products were sequenced and compared to other mosquito densovirus strains. Amplified DNA were cleaned using spin columns (Promega Wizard PCR Preps) and cloned into a pGEM-T vector (Promega). Plasmids were extracted (Wizard Minipreps, Promega) and sequenced in both directions using primers T7 and SP6 (Ampli*Taq* DNA polymerase, FS, Applied Biosystems on an automated sequencer, ABI 377).

Statistical analysis

Analysis of densovirus infection data used non-parametric methods because of the high proportion of zero counts. Infection rates in each species and stratified by site and season were compared using the Mann-Whitney U, Kruskal-Wallis and chi-square tests. Categories were collapsed for chi-square tests when it was necessary because of low expected counts, and Yates' correction was made in cases with only one degree of freedom. The association between densovirus infection and environmental variables was analysed using Spearman rank correlation coefficient. Climate data for the month of adult capture and time-lagged by one and two months before the collection month were included in the analysis.

RESULTS

Densovirus infection in adult mosquitos

Amplified PCR products were confirmed as being densovirus by sequencing. The sequences obtained from both *An. minimus* species were identical at the DNA level, and could be easily aligned with genomic sequences of other known mosquito densoviruses.

A total of 15.4% (21/136) adult *An. minimus* species A and 14.5% (98/678) species C were PCR-

Table 1
Densovirus infection in *An. minimus* species A and C as measured by PCR assay. Figures given are the number of densovirus positive specimens / total tested.

	Indoor			Peridomestic			Forest		
Month	sp A	sp C	% pos[a]	sp A	sp C	% pos	sp A	sp C	% pos
January	*	3/26	11.5	*	5/32	15.6	*	0/49	0.0
February	0/3	2/20	8.7	1/3	4/19	22.7	0/1	0/9	0.0
March	0/4	1/16	5.0	1/7	0/20	3.7	0/1	1/8	11.1
April	0/2	0/17	0.0	0/5	4/23	14.3	0/3	0/10	0.0
May	0/3	1/13	6.3	1/2	3/18	20.0	0/1	6/11	50.0
June	1/3	2/21	8.3	1/3	4/17	25.0	2/3	16/21	75.0
July	2/3	6/23	30.8	1/4	3/16	20.0	0/4	2/18	9.1
August	0/6	0/23	0.0	0/2	0/15	0.0	0/7	0/19	0.0
September	3/4	7/21	40.0	2/5	7/13	50.0	0/5	4/20	16.0
October	0/4	3/20	12.5	1/6	2/18	12.5	0/6	0/15	0.0
November	2/6	3/18	20.8	0/5	0/18	0.0	2/7	1/17	12.5
December	0/5	2/19	8.3	0/6	4/18	16.7	0/7	2/17	8.3
Total	8/43	30/237	12.7	9/48	36/227	16.7	4/45	32/214	15.2

[a]Percent DNV-positive, both species A and species C combined.
*Data not available

Table 2
Densovirus infection as measured by PCR assay in *An. minimus s.l.* larvae collected from February to December 1997 in Ban Phu Toei, Kanchanaburi Province, Thailand. Figures given are number of PCR positive specimens / total tested, with percent positive in brackets.

Month	Site A	Site B	Site C	Site D	All sites
February	1/4 (25.0)	0/5 (0.0)	0/3 (0.0)	0/3 (0.0)	1/15 (6.7)
March	1/4 (25.0)	*	0/5 (0.0)	3/5 (60.0)	4/14 (28.6)
April	2/5 (40.0)	0/3 (0.0)	0/2 (0.0)	1/2 (50.0)	3/12 (25.0)
May	0/5 (0.0)	0/3 (0.0)	0/4 (0.0)	0/5 (0.0)	0/18 (0.0)
June	3/8 (37.5)	0/4 (0.0)	0/3 (0.0)	0/2 (0.0)	3/17 (17.7)
July	5/6 (83.0)	2/3 (66.7)	0/4 (0.0)	0/4 (0.0)	7/17 (41.2)
August	1/8 (12.5)	1/4 (25.0)	0/4 (0.0)	0/4 (0.0)	2/20 (10.0)
September	3/5 (60.0)	0/2 (0.0)	3/4 (75.0)	0/6 (0.0)	6/17 (35.3)
October	2/6 (33.0)	*	2/4 (50.0)	2/7 (28.6)	6/17 (35.3)
November	3/8 (37.5)	*	1/5 (20.0)	1/5 (20.0)	5/18 (27.8)
Dececmber	3/7 (42.9)	*	1/5 (20.0)	1/5 (20.0)	5/17 (29.4)
Total (% positive)	24/66 (36.4)	3/24 (12.5)	7/43 (16.3)	8/48 (16.7)	42/223 (18.8)

Data from Site A, B and C combined. *No larvae collected because site was dry.

positive for densovirus (Table 1). Median infection prevalence was not statisitcally different between the two members of the species complex at any of the collection locations (indoor: p=0.479; peridomestic: p=0.623; forest: p=0.176, by Mann-Whitney U test). Consequently, data from both members of the complex were combined for further statistical analysis.

Densovirus infection frequency in *An. minimus s.l.* was not significantly different among the three collection locations (χ^2=1.02, df=2, p=0.60). Overall infection rates were 13.0% in mosquitos captured at the indoor site, 17.1% at the peridomestic site, and 15.2% at the forest site. However, there was significant temporal heterogeneity in infection frequency at all collection locations (indoor: χ^2=33.21, df=11, p<0.001; peridomestic: χ^2=28.71, df=11, p<0.004; forest: χ^2=108.38, df=11, p<0.001). Densovirus infection peaked in October, during the rainy season, in mosquitos captured at the indoor (40.0%) and peridomestic (50.0%) locations, and in June, during the hot season, in those from the forest location (75.0%). Furthermore, no densovirus-positive mosquitos were captured at the forest location during five of the months sampled. In contrast, there were only two months when no densovirus-positive mosquitos were encountered at the indoor and peridomestic locations.

Relationship of environmental variables to densovirus infection

The proportion of densovirus-positive *An. minimus s.l.* captured each month at the indoor location showed a significant positive correlation with the amount of rainfall recorded two months previous to the collection month (r=0.68, n=12, p<0.02, Spearman rank correlation coeffiicient). No statistically significant associations between densovirus infection and environmental variables were noted at the other sampling locations. Densovirus infection in *An. minimus s.l.* from the peridomestic site was negatively associated with the maximum daily rainfall in the month of collection (r=– 0.56, n=12, p<0.06); this relationship was marginally significant. Infection prevalence in mosquitos from the forest site showed a modest positive correlation with the mean temperature recorded in the previous month (r=0.49, n=12, p=0.11) and with rainfall in the previous month (r=0.44, p=0.15).

Densovirus infection in *An. minimus* larvae

An. minimus s.l. larvae were collected monthly from four different habitats. Larvae were not obtained from the swampy pool habitat, Site B, in March or October through December, because the site was dry. In addition to *An. minimus s.l.*, both *Culex* and *Aedes* larvae were found at Site A, a shallow stream located the closest to human dwellings of the four habitats that were sampled. At Site B, *Armigeres*, *Culex* and *Anopheles* were collected.

A total of 173 *An. minimus s.l.* larvae were PCR screened for densovirus infection. Site A was the only habitat from which densovirus-in-

fected larvae were collected consistently throughout the year (Table 2). At this site, there was only one month (May) when no densovirus-positive larvae were collected. In contrast, infected larvae were recovered from the other habitats in fewer than half of the months sampled. Average infection rates in larvae collected from each habitat ranged from 12.5% (Site B) to 36.4% (Site A). When data from all larval sites were combined, monthly infection prevalence showed a significant positive association with the number of rainy days recorded two months previous to the larval collection ($r=0.64$, $n=11$, $p<0.04$, Spearman rank correlation coefficient).

Densovirus infection in adult *An. minimus s.l.* was compared to larval infection rates in the month of adult capture, as well as to larval infection rates that were time-lagged by one and two months. The four larval sampling sites were combined to get an average monthly infection rate for *An. minimus s.l.* larvae in the study area. Monthly densovirus infection prevalence in adult *An. minimus s.l.* from the forest and peridomestic locations showed a significant negative relationship with larval infection in the previous month (peridomestic: $r = -0.67$, $n=11$, $p<0.03$; forest: $r = -0.70$, $n=11$, $p<0.02$; Spearman rank correlation coefficient). At the indoor site, the relationship between densovirus infection in adult mosquitos and larval infection in the previous month, although not statistically significant, was also an inverse one ($r = -0.25$, $n=11$, $p=0.46$).

DISCUSSION

This is the first report of natural densovirus infection in *Anopheles*. Results from the PCR-screening show densovirus infection is common in the *Anopheles minimus* species complex, averaging 15.4% in species A and 14.5% in species C. Both members of the complex appear to be equally susceptible to the virus, with no significant difference in infection frequency in adult mosquitos. Infection rates in *An. minimus s.l.* are lower than have been reported from surveys of densovirus in *Aedes aegypti* in Thailand, which range from 20% to 100% (Kittayapong *et al*, 1999). Several explanations may account for this difference. *An. minimus s.l.* may be less susceptible to densovirus infection than *Ae. aegypti*. Alternatively, densovirus infection may be more pathogenic to *An. minimus s.l.*, resulting in higher larval mortality and thus fewer infected survivng adults. Lastly, *An. minimus s.l.* may experience less larval-stage exposure to the virus than *Ae. aegypti* due to the nature of their larval habitat. *An. minimus s.l.* oviposit in streams and pools with good water circulation. In contrast, *Ae. aegypti* generally oviposit in water containers where densovirus may accumulate. Preliminary results from laboratory experiments suggest that *An. minimus s.l.* are equally susceptible and experience similar larval mortality as *Ae. aegypti* when reared in containers containing an oral suspension of *ATh*DNV (Rwegoshora, unpublished data). These data suggest that envirnomental factors likely account for the differences in infection prevalence between these two species in nature.

Our study used morphological keys to distinguish between the two members of the *An. minimus* species complex. Classification based on morphology alone, in particular the presence or absence of humeral pale spots on the wings, will correctly identify species C in 95% of instances and species A in 73% of instances. Since some adult mosquitos may have been identified incorrectly, it is possible that actual densovirus infection prevalence in each species may differ slightly from that we report here. However, since misclassification is assumed to be random with regard to densovirus infection status, conclusions regarding both *An. minimus* species' susceptibility to the virus are not likely to differ.

This is the first systematic temporal survey of natural densovirus infection in a mosquito species. Our data shows pronounced seasonal variation in densovirus infection in *An. minimus s.l.* Although overall infection frequency did not vary among mosquitos captured at the three locations, the timing of peak infection prevalence did, indicating some spatial heterogeneity in infection. Mosquitos captured at the indoor and peridomestic sites, located less than 50 m apart, exhibited similar trends in monthly infection prevalence, with peak infection rates occurring during the rainy season in September. In contrast, densovirus infection in *An. minimus s.l.* captured from the forest site, over 300 m distant, peaked earlier in the year during the hot season months of May and June. Furthermore, there were five months during which all forest-caught mosquitos were densovirus-negative, compared to only two months at the indoor and peridomestic sites. *An. minimus s.l.* captured at the two sites near human dwellings are likely to have shared the same larval habitat, perhaps even the same pool, and thus to have been exposed to

similar seasonal variation in densovirus concentration. Forest-caught adult mosquitos may have undergone their larval development in a distinct habitat that experienced different fluctuations in virus concentration.

Densovirus infection in adult *An. minimus s.l.* from the indoor site was strongly associated with the amount of rainfall two months before collection; however, infection in *An. minimus s.l.* caputred at the peridomestic and forest locations showed only a moderate association with climatic factors. In contrast, overall larval infection rates in the study area were strongly correlated with high rainfall in the month before larval collection. As the majority of densovirus infection is believed to be acquired during the larval stage (Barreau *et al*, 1997), the stronger relationship between climate and larval infection rates is reasonable. The amount of densovirus in the environment is likely to be influenced by a complex interplay of climate, larval density, species composition and other factors. Furthermore this relationship is likely to vary depending on the microhabitat. For example, high rainfall in some areas may wash densovirus out of the environment, while in others it may actually introduce the virus into a previously uninfected pool.

Our data suggest that the densovirus infection rate in adult *An. minimus s.l.* declines when virus availability in the larval habitat increases. Interestingly, infection in adult mosquitos from the peridomestic and forest sites showed a significant inverse association with infection in larvae in the month before collection. High larval infection rates are likely a consequence of an elevated virus concentration in the larval habitat, and as such may be associated with increased larval mortality. If fewer infected larvae survive, the frequency of infection in the adult population may be expected to decrease. In contrast, if virus concentration in the larval habitat is low, overall larval infection rates may be reduced but more infected individuals may survive, thereby increasing the frequency of infection in the adult population.

Densovirus infection in *An. minimus s.l.* may be a result of horizontal "contamination" from *Ae. aegypti*. *Aedes* larvae were observed sharing the same habitat as *An. minimus s.l.* at one of the larval collection sites that was located close to human dwellings. Laboratory data demonstrate that *An. minimus s.l.* are susceptible to infection with the Thai strain densovirus *ATh*DNV (Rwegoshora, unpubished data). In eastern Thailand, densovirus infection in *Ae. aegypti* field populations was shown to be continually maintained over a six month period (Kittayapong *et al*, 1999). In areas where *Ae. aegypti* and *An. minimus s.l.* are sympatric, climatic factors such as high rainfall may serve to expand the larval habitat of *Ae. aegypti* so that greater overlap between the two species occurs. Strong seasonal fluctuations in *Ae. aegypti* populations have been noted in some parts in Thailand, where increased rainfall is believed to provide additional larval habitats for this species (Mogi *et al*, 1988). Thus *Aedes* may actually serve as a "bridging" species, introducing densovirus into anopheline larval habitats during certain times of the year.

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