TITLE: *Armigeres subalbatus* colonisation of damaged pit latrines: A nuisance and potential health risk to residents of resettlement villages in the Lao PDR

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RUNNING HEAD (SHORT TITLE): Damaged latrines colonised by mosquitoes

ABSTRACT

During the resettlement of 6,500 persons living around the Nam Theun 2 hydroelectric project in Lao PDR, more than 1,200 pour-flush latrines were constructed. To assess the role of these latrines as productive larval habitats for mosquitoes, entomological investigations using CDC light traps, visual inspection and emergence trapping were carried out in over 300 latrines during the 2008-2010 rainy seasons. *Armigeres subalbatus* were nine times more likely to be found in latrines (mean catch = 3.09) compared with adjacent bedrooms (mean catch = 0.37; Odds Ratio (OR) = 9.08; 95% CI: 6.74 - 15.11) and mosquitoes were active in and around 59% of latrines at dusk. *Armigeres subalbatus* was strongly associated with latrines which had damaged or improperly sealed septic-tank covers (OR=5.44; 95% CI: 2.02 – 14.67; P<0.001). *Armigeres subalbatus* is a nuisance biter and a putative vector for Japanese encephalitis and dengue viruses. Dengue virus serotype 3 was identified from a single pool of non-blood fed female *Ar. subalbatus* using RT-PCR. Maintaining a good seal around septic tanks is a simple intervention to block mosquito exit/entry and contribute to vector control in the resettlement villages.

MAIN TEXT

Improved sanitation through the provision of latrines can contribute to a reduction in the incidence of diarrhoeal disease and soil-transmitted helminthic infections (Strunz *et al.*, 2014). Under Millennium Development Goal seven all United Nations member states agreed to halve the proportion of the global population without sustainable access to safe drinking water and basic sanitation. Between 1990 and 2012 nearly two billion people acquired access to improved sanitation facilities, including latrines, thus progress towards meeting this target has been made, though the target level of coverage will not be met by 2015.

Unfortunately, despite the extensive beneficial health outcomes associated with access to sanitation facilities, if latrines are not properly maintained they can form a productive habitat for vector mosquitoes. Mean catches in excess of 200 *Culex quinquefasciatus* per night have been reported for CDC light traps set in latrines in Dar es Salaam, Tanzania (Chavasse *et al.*, 1995), with reports of as many as 13, 000 mosquitoes from a single wet pit latrine on Zanzibar (Maxwell *et al.*, 1990).

As part of the resettlement programme of the Nam Theun 2 Hydroelectric Project (NT2), south-central Lao PDR, over 1,200 families (6,500 persons) were relocated and provided with newly constructed homes. Under the guidance of the millennium development goals, and with the desire to improve hygiene and living conditions, each resettled household was provided with a pour-flush latrine. We conducted investigations to determine whether latrines formed productive sources of mosquitoes in the resettlement villages within 3 years of their construction.

Latrines constructed by the NT2 resettlement programme were based on the pour-flush pit latrine design recommended by UNICEF (www.unicef.org/eapro/unprotected-EDEchapter7-2(1).pdf). They consisted of a water-sealed pan for defaecation, covered by a shelter built at ground level with concrete and wood-walls and a corrugated iron roof. Waste is delivered to the septic-tank through a pipe. The upper portion of the septic-tank was lined with a concrete ring of 80cm diameter with a circular concrete cover placed on top of the septic-tank and sealed with cement. A plastic ventilation pipe of 2cm diameter ran from the septic-tank to the top of the latrine wall (see Figure 1).

Informal discussions with the resettlement community during July 2008 (within one year of resettlement), indicated that latrines may have been acting as breeding sites for mosquitoes. Small holes (1-2cm diameter) beneath the concrete covers of septic-tanks were observed that may have allowed mosquitoes access to the wastewater inside. During September 2008 (late rainy season) a preliminary study was conducted in the bedrooms and latrine huts of 12 resettled households in Nakai Tai village (17°45’04.3” N, 105°06’32.8” E, 553 m elevation). The study aimed to determine which species of mosquito were collected in bedrooms of resettlement houses raised on stilts approximately 2.5m above ground, as well as in ground-level latrine huts of the same houses. CDC light traps (John W. Hock Co. Gainesville, FL) were set at dusk (between 18:00h and 19:00h) and collected between 07:00h and 08:00h the following morning. Over successive four-nightly intervals, each study house was sampled for three nights in the bedroom and once in the latrine. Traps in bedrooms were positioned at the foot end of an occupied bed, with the light suspended 1.5 m above the floor. Residents of all study houses were using insecticide-treated bed nets (B-52 Golden Horse Brand, Netto Manufacturing Co. Ltd., Thailand). In latrine huts the traps were suspended from the roof, 1 m distant from the latrine pan, with the light at 1.5 m above ground level. On any given night, nine bedrooms and three latrines were sampled concurrently. Over 19 nights, a total of 224 trap nights were successfully completed (4 traps failed and these data were excluded from further analysis).

Between August and September 2009 an observational study was conducted in and around the latrines of 50% of houses in three resettled villages (N=205 latrines): Done (17°40’07.1” N, 105°15’24.2” E, 551 m), Nakai Tai and NongBouaKham (17°49’15.8” N, 105°02’57.3” E, 544 m). In each village the latrine of every second house along a pre-determined walking route, was subject to a visual inspection at dusk. Each latrine was inspected for five minutes by a two-person team in order to record the presence or absence of mosquitoes flying around the hut or near the septic tank of the latrine. The presence of vegetation around the latrine hut, as well as any visible damage to the septic-tank cover, was recorded.

After stratifying by household using a Mantel-Haenszel chi-square test, the relative abundance of *Armigeres* mosquitoes caught in CDC light traps during 2008 was found to be significantly higher in latrines than bedrooms. *Armigeres subalbatus* formed 51.7% of the total catch in latrines (N = 317 females of all species, 53 trap nights), compared with 11.4% of the total catch in bedrooms (N = 551 females, 171 trap nights, $\chi^2 = 165.0$, $P < 0.001$). Odds of capturing *Ar. subalbatus* were 9.08 times higher in a latrine than in a bedroom (95% CI: 6.74 – 15.11) and the mean catch in latrines (mean

3.09, 95% CI: 2.30 – 3.89) was more than 8 times higher than in bedrooms (mean 0.37, 95% CI: 0.27 – 0.47) (GEE with negative binomial distribution and repeated measure for household, risk ratio (RR) = 8.54, 95% CI: 5.58 - 13.08, P < 0.001) (see figure 2). Although the relative abundance of Japanese encephalitis vectors (including *Culex tritaeniorhynchus, Cx. quinquefasciatus, Cx. vishnui, Cx. gelidus, Cx. fuscocephala* and *Cx. bitaeniorhynchus*) and putative malaria vectors (any anopheline) differed between latrines and bedrooms, mean catch sizes for these groups of species did not vary significantly between the two locations (figure 2).

During the subsequent observational study in three villages, mosquitoes were observed flying in and around 59% of 205 latrines at dusk. Damage to 17.6% of septic tanks was observed (N damaged = 36) and logistic regression analysis indicated that mosquitoes were more than five times as likely to be found in or around latrines with damaged or improperly sealed tanks compared with latrines that had intact tanks (OR = 5.44, 95% CI: 2.02 - 14.67, P < 0.001).

The results of these initial studies confirmed that mosquitoes were associated with latrines in the resettlement villages, but further investigations were needed to determine whether mosquitoes were newly emerging from the septic-tanks or merely resting in the dark, humid environments provided by the tanks and latrine huts.

During May 2010 (early rainy season, two years post-resettlement) sampling using CDC light traps took place in a randomly selected 40% of latrine huts in Nakai Tai village (N = 79 latrines) in order to provide estimates of mosquito density and to identify latrines with the highest mosquito catch sizes. The 36 latrines with the greatest mosquito catches were selected for subsequent sampling using emergence traps in order to identify exit points for newly emerged adults. Exit points were presumed to also form entry points for females searching for an oviposition site.

Three types of emergence trap were used during the third stage of the study (see figure 1): (i) whole hut enclosed with the ventilation pipe open, (ii) whole hut enclosed with ventilation pipe sealed, and

(iii) an emergence trap enclosing only the cover of the septic-tank. Each of the 36 latrines was sampled over three consecutive nights, using a different type of trap each night. Any indoor-resting mosquitoes were removed from the latrine hut before setting the emergence trap. The presence of vegetation around the latrine, water for flushing, and any damage to the waste delivery pipe and/or septic-tank was recorded. A door sealed with cord ties was incorporated into the design of the large emergence trap so that people could access the latrine during the night. Two of each type of trap were set before dusk each night (N = 6 traps per night) and mosquitoes were collected from all traps between 07:00h and 08:00h the morning after the trap was set.

Over 108 trap nights a total of 1,866 mosquitoes (59.5% female, of which 98.5% were unfed and 1.5% blood fed or gravid) were collected from the three types of emergence trap. *Armigeres subalbatus* comprised 88.7% of females and 91.1% of males, whilst *Culex quinquefastciatus* comprised 10.8% of the female catch and 8.9% of males. Although mosquitoes of either sex or species were found in at least one emergence trap at 34 of the latrines, the total catch was highly skewed; with 5 latrines producing most of the total *Ar.subalbatus* catch.

Analysis using a negative binomial regression model with household-level clustering, revealed that fifteen times more female *Ar. subalbatus* were caught in emergence traps positioned directly over the septic-tank cover, compared with traps positioned over the latrine hut with the ventilation pipe open (RR = 15.25, 95% CI: 3.17-73.36, P = 0.001). A maximum of 496 females were captured in one night in this type of emergence trap. Fourteen times more males were collected from septic-tank emergence traps, compared with traps covering latrine huts with open ventilation pipes (RR = 13.75, 95% CI: 2.7 – 70.13, P = 0.002). This result indicated that ventilation pipes did not form an access point to septic-tanks for mosquitoes in the resettlement villages.

Despite the wide variation in catch sizes, damaged septic-tank covers (N = 6) were significantly associated with female *Ar. subalbatus* catch sizes that were almost five times greater than catches from undamaged septic-tanks (N = 21) (RR = 4.82, 95% CI: 1.31-17.72, P = 0.019). Males were also more likely to be caught in emergence traps at latrines with damaged septic-tanks compared with tanks that had intact covers, but this difference was not statistically significant (RR = 3.49, 95% CI: 0.70 – 17.33, P = 0.122). In latrines where the tank cover was buried completely below the ground (N = 9) the likelihood of capturing female *Ar. subalbatus* was substantially reduced, compared with latrines where the cover was visible but intact (RR = 0.07, 95% CI: 0.02 – 0.31, P = 0.001). The same was true for males, though the variation in catch sizes was much greater (RR = 0.13, 95% CI: 0.02 – 0.86, P = 0.035). The absence of water for flushing the latrine was not associated with a reduced likelihood of finding mosquitoes in an emergence trap.

Armigeres subalbatus captured in latrine CDC light traps were pooled and frozen at -80°C for further virological analysis given their putative role in flavivirus transmission. A total of 1,175 specimens (983 females, 192 males) were sorted and pooled by date and capture site. A total of 101 pools (females: 70; males: 31) were processed for total RNA extraction as previously described (Pagès *et al.*, 2009). Purified RNA was submitted to a first screening by a pan-*flavivirus* RT-nested PCR (Sánchez-Seco *et al.*, 2005). Among the pools tested, five were positive for the presence of *flavivirus* sequences (4.9% of pools; females: 4 positive; males: 1 positive pool). All positive samples were submitted to a second battery of specific real time RT-PCR to attempt virus identification, including a standard approach to test samples for dengue virus and to determine the virus serotype (Lao *et al.*, 2014). Dengue virus serotype 3 was identified using RT-PCR from a single pool of non-blood fed female *Ar. subalbatus*. The four remaining pools were negative for all specific RT-PCR tested viruses (dengue; West Nile; Japanese encephalitis). Subsequently, a dengue virus 3 serotype was isolated from the RT-PCR positive pool homogenate following inoculation onto C6/36 cells. This viral isolation formally excludes a possible RT-PCR cross contamination. Although this result does not demonstrate the direct role of this species as a vector of dengue, it at least demonstrates the active replication of dengue 3 virus in *Ar. subalbatus* tissues.

During November 2011 (end of the rainy season) a follow-up study was conducted in Done village in order to investigate whether burying septic-tank covers beneath a layer of earth could prevent them from becoming oviposition sites. Eighty households were randomly selected for inclusion in the study and emergence traps were positioned over the septic-tank cover of each latrine for one night. Tank covers were classified as: completely covered by soil (buried), visible and intact, visible and damaged or the tank location could not be found and an emergence trap was not set. In agreement with the findings of the previous year, when tanks were completely buried below ground (N = 10 latrines) no male or female *Ar. subalbatus* were caught in emergence nets. Where tank covers were damaged, female catch rates were 78 times higher than in intact tanks (RR = 77.6, 95% CI: 29.6 – 203.0, P < 0.001) and male catch rates were 104 times higher (RR = 103.5, 95% CI: 36.6 – 292.9, P<0.001) than in intact tanks.

Discussion:

During the course of a number of studies between 2008 and 2011 it was demonstrated that if septic-tank covers are damaged, even newly constructed latrines can be highly productive habitats for *Ar. subalbatus* mosquitoes.

During the first year following latrine construction and population resettlement, *Ar. subalbatus* were found in latrine huts, but rarely in bedrooms. It is possible that the small volume of the latrine hut, or position low to the ground, might explain increased catch sizes due to the higher concentration of attractive odours in a room of smaller volume compared with a bedroom. Ground-level latrine huts may also have been more accessible when compared with bedrooms that were in houses raised above ground on stilts (Charlwood *et al.*, 2003; Lee *et al.*, 2006). However, this theory would imply that catch sizes of putative JE and malaria vectors would also be greater in latrines compared to bedrooms, which was not the case.

During the observational study, mosquitoes were seen flying around latrine huts at dusk, an observation which is in line with previous studies on mosquito time of emergence (de Meillon *et al.*, 1967). This supports the conclusion that these were newly emerged mosquitoes.

During the emergence trapping study a large number of males were captured (40.5% of the total catch), indicative that the catches represented emergence from a larval habitat, rather than captures from a resting site where females were digesting blood. It is assumed that the slight female bias was due to trapping of females which entered the septic tank to oviposit before the emergence trap was set. It is unlikely that septic tanks formed an important resting site as most captured females were unfed (only 1.5% were blood fed or gravid). Emergence trap catches were highest in traps that were positioned over damaged septic-tank covers, indicating that small gaps in the concrete cover formed the exit, and presumably entry point, for *Ar. subalbatus*. Trapping using large emergence nets enclosing the entire latrine hut with the ventilation pipe open, did not suggest that mosquitoes were exiting septic-tanks via this route.

While the vectorial status of *Ar. subalbatus* in the Lao PDR is not confirmed, Japanese encephalitis virus (JEV) has been detected in *Ar. subalbatus* from Taiwan and Yunnan Province, China (Chen *et al.*, 2000; Feng *et al.*, 2012; Liu *et al.*, 2013). After feeding *Ar. subalbatus* on a suspension of JEV which had been isolated from a sympatric region of Taiwan, 79% of females were found to have disseminated virus in the salivary glands (Chen *et al.*, 2000), a strong indicator that this mosquito could act as a vector. As JEV has been shown to circulate among the NT2 resettlement population (Hiscox *et al.*, 2010) the increased abundance of this non-conventional vector species could facilitate increased transmission of the disease. Among the pools of mosquitoes collected in the resettlement villages, nearly 5% were positive for flavivirus sequences, but direct identification of a virus species (i.e. dengue) was established from only one pool. A number of mosquito species, including *Ar. subalbatus*, have been reported to harbour either viral or insect-specific flavivirus sequences (Takhampunya *et al.*, 2014). Despite being negative for known flaviviruses by RT-PCR, the other panflavi-positive pools warrant further sequencing in this specific context where high *Ar. subalbatus* populations are coexisting with humans. Detection and isolation of dengue virus serotype 3 from a pool of non-blood fed females demonstrates the possible infection of *Ar. subalbatus* by dengue, but does not allow us to confirm its competence nor the role of this species as a vector for dengue virus. However, this aspect should be investigated more thoroughly to determine its putative epidemiological impact on dengue transmission in areas, or during periods where conventional vectors densities are naturally or artificially low. This will allow us to better determine the role of *Ar. subalbatus* as a possible vector.

In addition to the potential capacity of *Ar. subalbatus* to act as a disease vector in these villages, informal discussions with members of the community revealed that substantial levels of nuisance biting are experienced in latrines. If nuisance biting were to deter people from using latrines, the knock-on effect of mosquito breeding could be an increase in the transmission of soil-transmitted helminthic infections and diarrhoeal disease in the resettlement population.

The application of a floating layer of polystyrene beads has been used to control breeding of *Cx. quinquefasciatus* in soakage pits and wet pit latrines in Tanzania and India (Curtis *et al.*, 2002) and the use of this technique has been suggested for *Ar. subalbatus* (Sivagnaname *et al.*, 2005). Unfortunately application of this technique to latrines in the NT2 resettlement area would be hindered by the small size of openings (often only 1-2cm diameter) through which mosquitoes access the latrines. Concrete septic-tank covers are sealed in place and cannot be easily removed in order to apply the beads. An alternative, cheap and environmentally friendly approach to vector control would be to ensure that septic-tanks are fully covered with earth, thus blocking any small holes in the septic-tank covers. During our studies of 2010 and 2011, septic-tanks that were buried below the ground were devoid of mosquito breeding. Household owners could do the covering at the time of installation, or at a later date, and minimal training would be needed to explain this simple process to the community.

In conclusion, despite large improvements in health to be gained through the provision of sanitation facilities, poor maintenance can lead to pour-flush latrines becoming highly productive mosquito habitats. Simply covering septic tanks with a layer of soil should dramatically reduce the number of mosquitoes produced, but field data is needed in order to validate this recommendation. As the NT2 resettlement programme followed a latrine design which was recommended by UNICEF, the Asian Development Bank and the World Bank, the implications of these findings could be far reaching as similar latrine designs are used all over the world.

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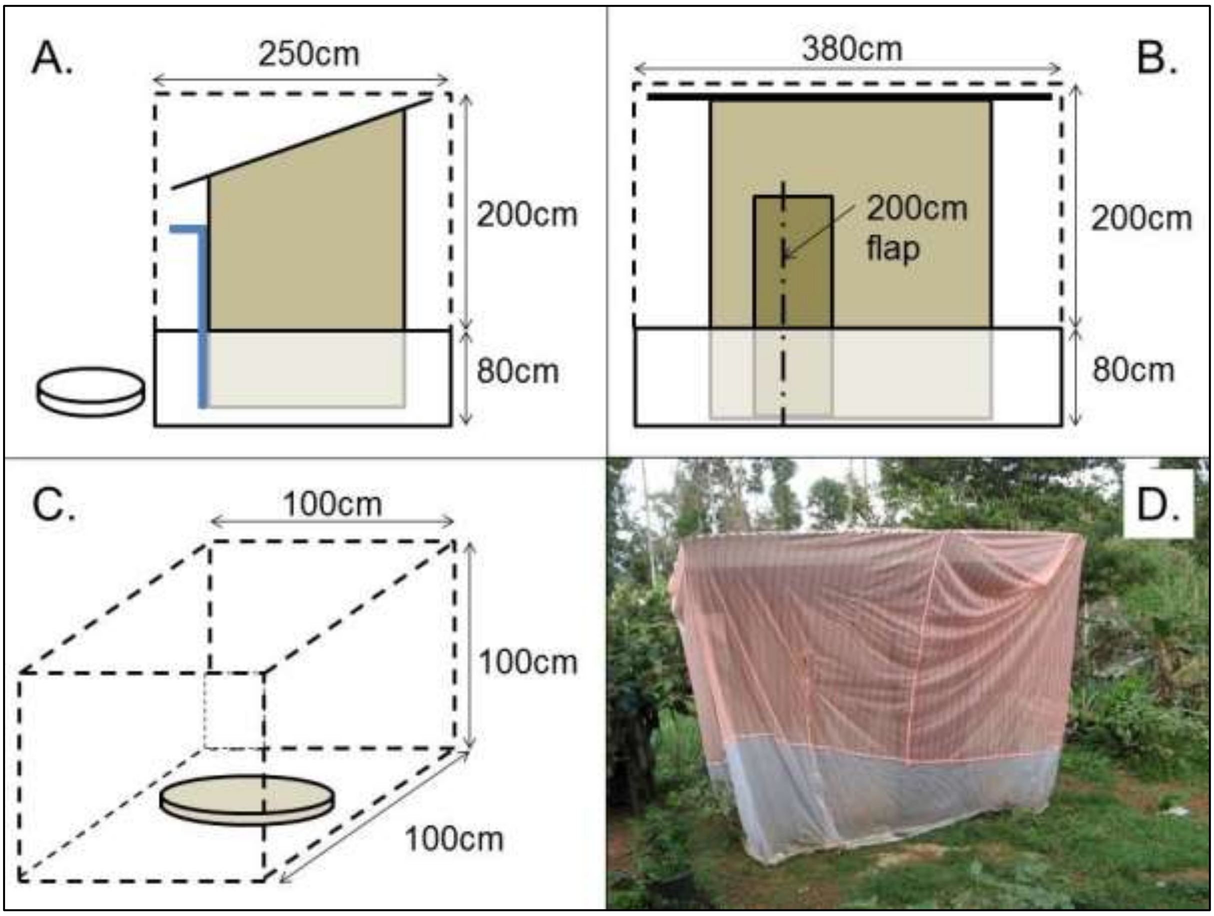
FIGURE LEGENDS

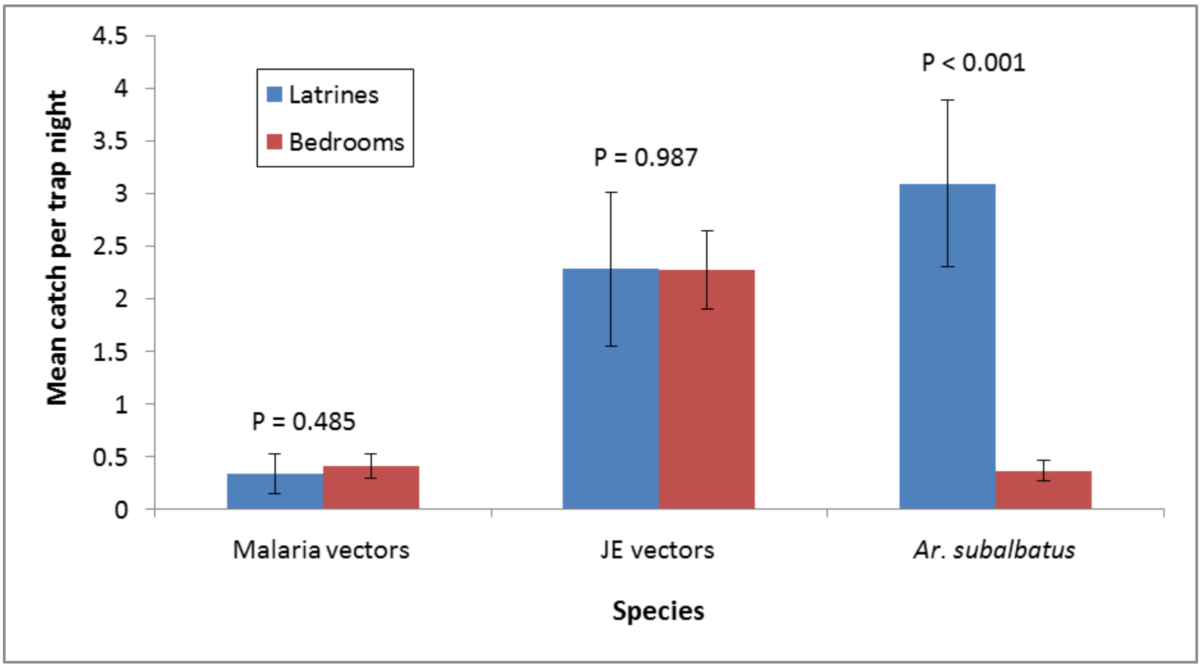
Figure 1: Emergence traps used to capture mosquitoes in latrine huts and septic-tanks. A: side section of a latrine hut with blue ventilation pipe enclosed in an emergence trap and the septic-tank outside the emergence trap, B: front section of a latrine hut covered in an emergence trap showing the door flap used to keep the trap closed but allow access to the latrine hut, C: emergence trap covering a septic-tank, D: photograph of the same view shown in B, a latrine hut covered by an emergence trap.

Figure 2: Mean CDC light trap catches for putative vectors of malaria (any anopheline), Japanese encephalitis (including *Culex tritaeniorhynchus, Cx. quinquefasciatus, Cx. vishnui, Cx. gelidus, Cx. fuscocephala* and *Cx. bitaeniorhynchus*) and *Armigeres subalbatus* in latrines and bedrooms of Nakai

Tai village. Error bars indicate 95% confidence intervals for the mean, P-values are for the difference between in mean catches between bedrooms and latrines for each species, N = 53 trap nights in latrines and 171 in bedrooms.

FIGURES (to be uploaded as separate files during submission process)




Mean catch per trap night
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Latrines
Bedrooms
P = 0.485
P = 0.987
P < 0.001
Malaria vectors
JE vectors
Ar. subalbatus
Species