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## RESEARCH ARTICLE

# Serological biomarker for assessing human exposure to *Aedes* mosquito bites during a randomized vector control intervention trial in northeastern Thailand

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**Data Availability Statement:** The data underlying the results presented in this paper are made publicly available following principles that data should be Findable, Accessible, Interoperable, and Reusable (FAIR, <https://www.force11.org/group/fairgroup/fairprinciples>) and regulations of the project owner, the Norwegian University of Life Sciences, that research data must be archived in approved national or international archives. The data used in this publication is archived at the

## Abstract

### Background

*Aedes* mosquitoes are vectors for several major arboviruses of public health concern including dengue viruses. The relationships between *Aedes* infestation and disease transmission are complex wherein the epidemiological dynamics can be difficult to discern because of a lack of robust and sensitive indicators for predicting transmission risk. This study investigates the use of anti-*Aedes* saliva antibodies as a serological biomarker for *Aedes* mosquito bites to assess small scale variations in adult *Aedes* density and dengue virus (DENV) transmission risk in northeastern Thailand. Individual characteristics, behaviors/occupation and socio-demographics, climatic and epidemiological risk factors associated with human-mosquito exposure are also addressed.

### Methods

The study was conducted within a randomized clustered control trial in Roi Et and Khon Kaen provinces over a consecutive 19 months period. Thirty-six (36) clusters were selected, each of ten houses. Serological and entomological surveys were conducted in all houses every four months and monthly in three sentinel households per cluster between September 2017 and April 2019 for blood spot collections and recording concurrent immature and adult *Aedes* indices. Additionally, the human exposure to *Aedes* mosquito bites (i.e., Mosquito Exposure Index or MEI) was estimated by ELISA measuring levels of human antibody response to the specific Nterm-34 kDa salivary antigen. The relationships between the MEI, vector infestation indices (adult and immature stages) and vector DENV infection were

Norwegian Center for Research Data (NSD), a national archive and center for research data. Data can be accessed without specific permissions by filling in and submitting a Data Access Form at <https://nsd.no/nsd/english/order.html>, referring to the project ID no. NSD2864 and project name "Determination of entomological indices to assess dengue transmission, predict dengue outbreaks and evaluate vector control interventions, 2019", short name: DENGUE-INDEX.

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**Competing interests:** The authors have declared that no competing interests exist.

evaluated using a two-level (house and individual levels) mixed model analysis with one-month lag autoregressive correlation.

## Results

There was a strong positive relationship between the MEI and adult *Aedes* (indoor and outdoor) density. Individuals from households with a medium mosquito density (mean difference: 0.091,  $p < 0.001$ ) and households with a high mosquito density (mean difference: 0.131,  $p < 0.001$ ) had higher MEI's compared to individuals from households without *Aedes*. On a similar trend, individuals from households with a low, medium or high indoor *Aedes* densities (mean difference: 0.021,  $p < 0.007$ , 0.053,  $p < 0.0001$  and 0.037,  $p < 0.0001$  for low, medium and high levels of infestation, respectively) had higher MEI than individuals from houses without indoor *Aedes*. The MEI was driven by individual characteristics, such as gender, age and occupation/behaviors, and varied according to climatic, seasonal factors and vector control intervention ( $p < 0.05$ ). Nevertheless, the study did not demonstrate a clear correlation between MEI and the presence of DENV-infected *Aedes*.

## Conclusion

This study represents an important step toward the validation of the specific IgG response to the *Aedes* salivary peptide Nterm-34kDa as a proxy measure for *Aedes* infestation levels and human-mosquito exposure risk in a dengue endemic setting. The use of the IgG response to the Nterm-34 kDa peptide as a viable diagnostic tool for estimating dengue transmission requires further investigations and validation in other geographical and transmission settings.

## Author summary

*Aedes* mosquitoes and the viruses they transmit are major public health concerns for over half of the global human population. However, the quantitative relationships between virus transmission and vector mosquito infestation remain unclear despite numerous indicators used to estimate transmission risk and predict dengue outbreaks. The aim of this study is to investigate the use of a salivary biomarker to assess the small-scale variation in human exposure to *Aedes* bites and the risk of dengue infection in the context of a vector control intervention in northeastern Thailand. A cohort of 539 persons visited every four months, including 161 individuals visited monthly, were recruited for routine serological and concurrent household entomological surveys during 19 consecutive months follow-up. Antibody response to *Aedes* bites was measured by enzyme-linked immunosorbent assays to assess the mosquito exposure index (MEI) and association with the *Aedes* adult and immature abundance as well as the presence of dengue virus (DENV) in adult mosquitoes (transmission risk). Additionally, the individual (cohort), climatic, and vector control intervention risk factors associated with MEI are explored. This study demonstrates that the MEI was strongly related to household adult *Aedes* density, particularly indoors resting mosquitoes. Additionally, the MEI was influenced by individual characteristics (i.e., person age, gender, staying indoors), and varied according to seasons and intervention. Nonetheless, no clear relationship between MEI and dengue transmission risk (i.e., vector infection) was detected. This study demonstrated the potential usefulness

of the MEI to assess heterogeneity in adult *Aedes* infestation indices that could assist public health authorities to rapidly identify mosquito “hot spots” and the timeliness of effective vector control interventions.

## Introduction

*Aedes aegypti* (L) and *Aedes albopictus* (Skuse) are vectors of important human viral pathogens including dengue, yellow fever, chikungunya and Zika. In Southeast Asia, dengue fever is widespread and accounts for around 70% of the total clinical dengue cases reported globally [1,2]. Since the first report of dengue infection in Thailand in 1949 [3], dengue incidence has dramatically increased in line with expanding urbanization. With all four virus serotypes and both major mosquito vectors present in the country around 20,000 cases are reported yearly [4]. Despite an affordable, universal primary health coverage system and an organized, nationwide dengue prevention program, the burden of dengue in Thailand is estimated to cost the equivalent of at \$290 million (USD) each year [5].

In northeastern Thailand, dengue fever represents major public health concerns with thousands of clinical cases each year [6]. To prevent secondary transmission in communities, when a dengue case is detected, insecticide treatment using adult space spray is mandated within 24 hours in attempt to rapidly eliminate virus-infected vectors, surrounding its home setting [7]. In parallel, basic entomological surveillance is carried regularly by one of the 22 regional Offices of Diseases Prevention and Control (ODPCs) to monitor *Aedes* vector infestations [7]. In Thailand standard entomological indices are used to estimate transmission risk that guide the choice of vector control interventions [8]. While, some studies have shown positive associations between various entomological indices and disease transmission risk [9,10], other investigations have demonstrated only weak relationships [11–13]. Most of the entomological indices used to monitor dengue vector infestations are based on measuring the presence of immature mosquito life stages [14]. However, immature stages typically present large mortality rates during development from egg to adult stage [15], thus larval indices do not provide an accurate or concurrent temporal-spatial information on the ‘productivity’ of containers regards actual *Aedes* adult production output [16]. Conversely, pupal indices have been proposed to assess vector infestation with higher accuracy [17,18] as pupae generally present very low mortality up to adult emergence and thus more relevant to estimate container productivity [19] and adult densities in a location [16]. Operationally, pupae collections remain difficult to implement on a routine basis because they are time-consuming (generally all pupae must be collected and counted) that requires additional entomological staff.

Adult mosquito collections have been used to estimate the risk of virus transmission [19,20], but they have also their limitations. Unlike malaria vector monitoring, human land-catching cannot be performed to collect *Aedes* mosquitoes due to the inherent ethical constraints and disease risks, as there is no preventive treatment nor effective vaccines for most of *Aedes*-transmitted diseases/pathogens (except yellow fever virus). Moreover, *Aedes* adults are most active during the day time, when most people are awake and can take some forms of protection against bites. As a consequence, *Aedes* females are often interrupted in the course of seeking a blood meal and can often feed on multiple hosts per gonotrophic cycle [21–23]. Other methods to sample adult *Aedes* include various versions of passive and active trapping devices (e.g., gravitraps, sticky traps, mechanical battery-operated aspirators, and mosquito electrocuting trap) [24], each presenting differing levels of efficiency [25]. However, they do not measure the inter-individual heterogeneity of exposure influenced by human attraction

exerted on mosquitoes and individual host behaviors (e.g., use of personal protections). Nevertheless, these capture methods are used as a proxy to estimate *Aedes* adult density in a specific area but they are not representative of actual level of contact (biting) exposure between human and vector [26]. This information is yet crucial to identify host population subsets at higher risk of exposure to dengue vector bites and to better estimate virus transmission risk.

An alternative to direct entomological indices for estimating the human exposure to mosquitoes is the measure of a host's antibody (Ab) response to mosquito saliva antigens [27–29]. During blood feeding process, mosquito saliva is initially injected into human skin to facilitate the blood intake and also acts as a vehicle for transmitting pathogens to the host [30]. Many salivary proteins are immunogenic and elicit an immune response including the production of specific antibodies (Ab) that can be detected by simple analytic tools and spectrophotometry [31–33]. Firstly developed for *Anopheles*, the vectors of malaria, so-called biomarkers of exposure based on anti-saliva Ab response have been used successfully to identify “hot spots” of vector presence and malaria transmission [34–36] along the Thailand-Myanmar border [34,37]. As far as *Aedes* genus is concerned, several other studies have shown that IgG response to salivary gland extracts from different *Aedes* species, such as *Ae. aegypti*, *Ae. polynesiensis*, *Ae. caspius* are reliable indicators of human-*Aedes* exposure in South-America [38,39], Pacific Islands [40], Africa [41] and Europe [31]. An *Ae. aegypti*-specific salivary peptide (Nterm-34 kDa) has been identified and the human IgG response to the Nterm-34 kDa antigen has shown good correlation with adult *Ae. aegypti* infestation indices in Benin [42] and Laos [43]. More recently, the Nterm-34 kDa salivary peptide successfully investigated the spatial heterogeneity of *Aedes* exposure in several urban districts of Senegal [44]. However, most of these *Aedes* serological studies estimated vector infestation through “indirect” (relative) indicators such as immature ‘*Stegomyia*’ (*Aedes*) indices and climatic factors, thus were unlikely to represent more accurate adult infestation that which is directly associated with virus transmission potential. Robust evidence of the relationships between the intensity of human immune response to a specific salivary biomarker, *Aedes* adult abundance, and dengue infective bite risk is needed to assess whether small scale variations in dengue transmission can be detected using this immunological tool. This is particularly relevant for measuring the impact of vector control interventions where entomological indices may lack the spatio-temporal accuracy and sensitivity to demonstrate control effectiveness [16,45,46].

The primary objective of this study was to assess the relationship between the intensity of the human IgG response to the Nterm-34kDa *Aedes* salivary peptide and selected entomological indicators of vector infestation and dengue infection risk in northeastern Thailand. This study took place within the context of a randomized controlled trial implemented over a consecutive 19-month period to evaluate the efficacy of an insect growth regulator tool for dengue transmission prevention [47,48]. Additionally, risk factors associated with human-vector contact in terms of individual human characteristics and behavior, local vector control practices, and the prevailing seasonal and climatic factors were addressed. To our knowledge, this is the first longitudinal study conducted to assess dengue transmission risk using a serological *Aedes* salivary biomarker. Hopefully, these findings will assist national authorities to improve the accuracy of dengue surveillance activities and contribute to strengthening the monitoring and evaluation of vector control programs in Thailand and elsewhere.

## Materials and methods

### Ethics statement

This trial was registered (ISRCTN, ISRCTN73606171) and approved by the Khon Kaen University Ethics Committee (KKUEC Record No. 4.4.01: 29/2017, Reference No. HE601221, 1

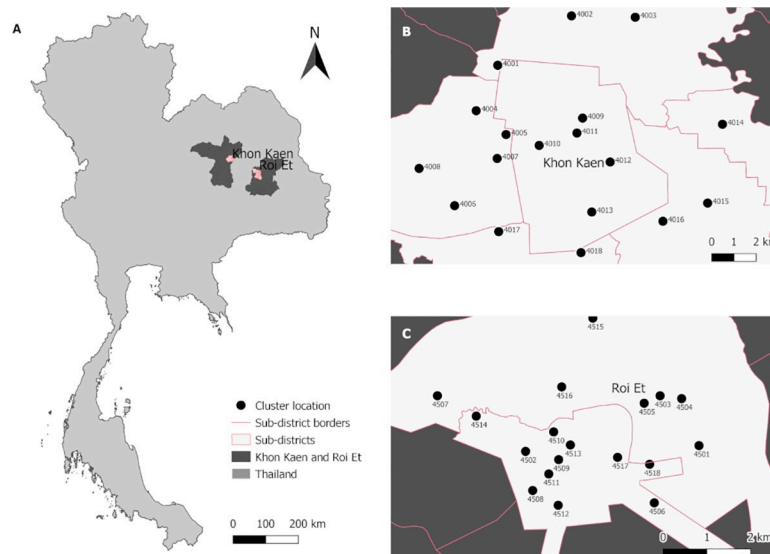
September 2017), the London School of Hygiene and Tropical Medicine Ethical Committee, UK (LSHTM Ethics Ref: 14275, 16 August 2017), and the Regional Committee for Medical and Health Research Ethics, Section B, South East Norway (REK Ethics ref: 2017/1826b, 03 March 2018). Each participant was informed about the intent of the study and asked to participate on a voluntary basis. In each household, the head of the house signed a consent form to allow periodic entomological inspection inside and outside their residence. Additionally, signed informed consent (or assent, if under 16 years old) were required each time blood samples were taken.

### Study sites

The study was conducted in six sub-districts in the city of Khon Kaen (KK), Khon Kaen Province, (N16.440236, E102.828272) and in two sub-districts within the city of Roi Et (RE), Roi Et Province, (N16.055637, E103.652417), in northeastern Thailand (Fig 1). In each city, 18 clusters of 10 households each were randomly selected for a total of 360 households under 19 months of follow-up.

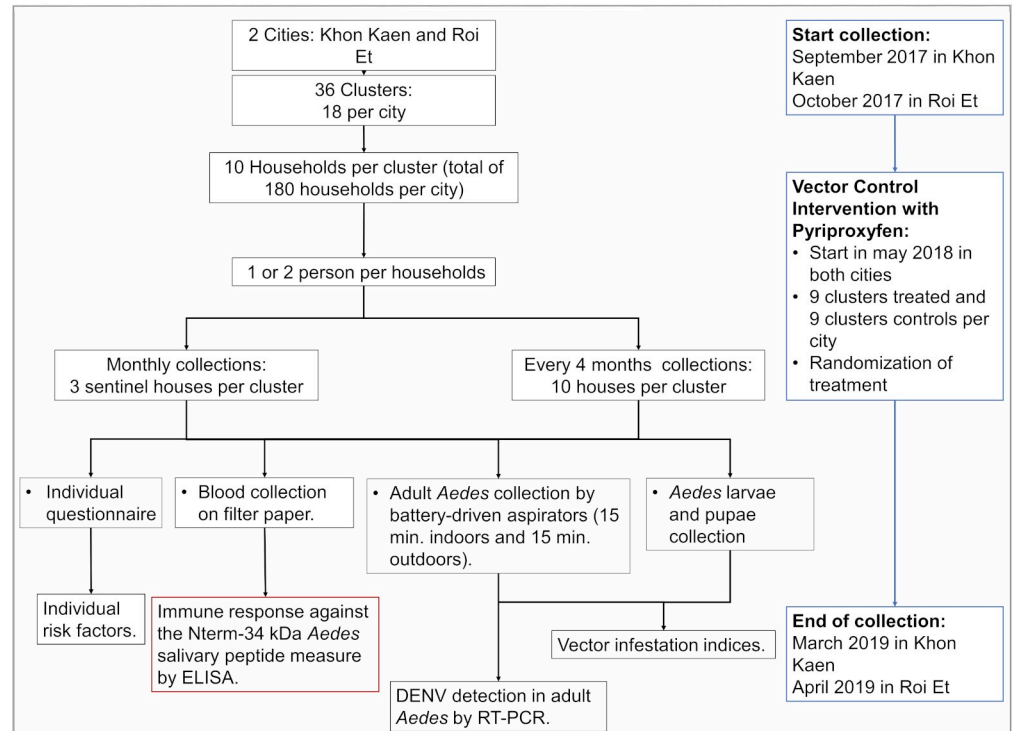
### Study design and settings

This study was conducted within the framework of a randomized control intervention trial to evaluate the efficacy of pyriproxyfen application (0.5% granule formulation) for dengue vector control [47,48]. The study was performed in Khon Kaen between September 2017 and March 2019 and between October 2017 and April 2019 in Roi Et (Fig 2). All households were visited every four months (except one time in RE between February 2018 and May 2018) to collect indoor and outdoor container-breeding *Aedes* (both *Ae. aegypti* and *Ae. albopictus*) larvae, pupae, adult resting mosquitoes, and blood samples from study volunteers living in randomly selected households. In addition, three sentinel houses per cluster were visited monthly for blood and entomological collections described previously. Following the initial 10 months of



**Fig 1. Map of study sites.** (A) represents Thailand and the provinces of Roi Et and Khon Kaen. (B) shows the location of the 18 clusters numbered from 4001 to 4018 in the city of Khon Kaen (KK Mueang District). (C) shows the location of the 18 clusters numbered from 4501 to 4518 in the city of Roi Et (RE Mueang District). Map of study sites was built using QGIS 3.10 software and shapefiles were obtained from the Humanitarian Data Exchange project [49] under the Creative Commons Attribution International 4.0 license (CC BY 4.0).

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**Fig 2. Study design flow chart.** RT-PCR: Reverse Transcriptase Polymerase Chain Reaction.

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baseline surveillance, the vector control intervention was distributed randomly in half of the study clusters, in June 2018. The household selection in the cities and the randomization of the intervention are described elsewhere [47,48]. The vector control intervention was the distribution of pyriproxyfen (0.5% granule formulation) into water-holding containers up to 0.01 mg/L active ingredient applied every four months in the treated clusters [47,48].

### Individual volunteer characteristics

In each participating household, at least one volunteer inhabitant was recruited in the study. When possible, we tried to recruit inhabitants spending most of their time at home. To ensure adequate representativeness of the entire target population, we recruited one adult and one child per house when feasible. In addition, a pecuniary retribution (50THB) for blood sampling was given to each participant. During each household visit, assigned trained Village Health Volunteers (VHV) interviewed and collected blood of each participating house member. Interview questions were relative to the general characteristics of the participant (i.e., age, gender), occupation(s) during the weekdays and weekends (e.g., at home; at work away from home; at school/college/university; at farm; others), in addition to normal activity and resting habits (i.e., primarily indoor, outdoor or equally indoor and outdoor). The travelling history within the previous 14 days and within the last three months was recorded.

### Blood sample collections

Blood samples (2 blood spots per participant, 10mm diameter each, approximately 150µl) were collected at the fingertips of the inhabitants recruited in the study using sterile lancets [50] and spotted on filter paper Protein Saver cards (Whatman, Maidstone, UK), air-dried, individually placed in plastic sealable bags and stored at room temperature at the Office of

Disease Prevention and Control 7 (ODPC7) until delivery to Khon Kaen University (KKU) and stored at 4°C.

## Entomological collections

At each household visit, the VHVs recorded the number of inhabitants in the household at the time of the survey. Houses were inspected for adult and immature *Aedes* both indoor and outside immediately surrounding the house. The total number of containers was recorded together with the number of wet containers at each household. A maximum of 20 larvae (preferably late stage instar) and all pupae were collected per infested container and stored in absolute ethanol at the ODPC7. Immatures and adults were identified to species-level using morphological keys [51,52], and sex was determined for adults. *Aedes* adult collections were performed using hand-held mechanical battery-powered aspirators [53] conducted 15 min each both indoors and outdoors. Adults were stored individually in labelled 1.5mL microcentrifuge tubes at -20°C and the house number and the location of collection (i.e., indoor/outdoor) was recorded.

Entomological data were used to construct several indices as described in Supporting information S1 Table and *Aedes* indices described hereafter include both *Ae. aegypti* and *Ae. albopictus*. At the cluster level, the Container Index (CI<sub>c</sub>) was calculated as the proportion of *Aedes* immature-positive containers per total wet containers inspected in all visited households at the time of survey. The cluster-wide Breteau Index (BI<sub>c</sub>) and the House Index (HI<sub>c</sub>) were calculated as the proportion of *Aedes* positive containers per 100 houses and the proportion of positive households visited, respectively. The cluster-level pupal indices, Pupae per House Index (PHI<sub>c</sub>) and the Pupae per Person Index (PPI<sub>c</sub>), represented the total number of pupae collected per household and per inhabitants in each visited household, respectively. The *Aedes* Index (AI<sub>c</sub>) and the *Aedes* indoor Index (AI<sub>in,c</sub>) at the cluster level represented the total number of female *Aedes* collected per inspected houses and the total number of female *Aedes* collected exclusively indoors, respectively.

## Detection of dengue virus in adult mosquitoes

The presence of dengue virus (DENV) in *Aedes* females was investigated in all captured adult mosquitoes, by pooling up to 10 individual abdomens of female *Aedes* together for RNA extraction and DENV detection by reverse transcriptase real-time polymerase chain reaction (RT-qPCR) [54]. For positive pools, the head and thorax of the corresponding individual mosquitoes were processed individually for DENV serotype detection according to Lanciotti *et al* protocol and adapted by our team to be run on RT-qPCR [54]. The proportion of DENV infected *Aedes* was calculated as the number of DENV infected individual *Aedes* divided by the number of tested *Aedes* females per house (AI DENV+) and per cluster (AI<sub>c</sub> DENV+), respectively.

## Climatic data

The Meteorological Department of Thailand provided climatic data routinely recorded from the meteorological stations located at the airport of each city [55]. Daily measures were used to derive the minimum and maximum air temperatures (°C), the percent relative humidity, and the rainfall (mm) between January 2016 to January 2020. For analysis, the mean maximum and minimum temperatures, mean percent relative humidity, and cumulative rainfall the previous two weeks before entomological collections were used to account for an estimated time-lag effect on vector population biology and transmission epidemiology.



### Mosquito Exposure Index (MEI)

The specific human IgG response to the Aedes Nterm-34kDa salivary peptide (Genepep, Saint Jean de Védas, France) was measured by an enzyme-linked immunosorbent assay (ELISA) as described previously [48,56]. This secreted salivary peptide was selected because it exhibits high antigenic properties and it is specific to *Aedes* genus, therefore allowing to specifically measure the immune response to *Aedes* bites alone [42]. Briefly, for each individual sampled, dried blood spots were cut using a one cm diameter hole punch and eluted in 400µl of Phosphate Buffer Saline (PBS) for 24h at 4°C. The resulting eluates were stored at -20°C until further processing. 96-well Maxisorp micro-assay plates (Nunc, Roskilde, Denmark) were coated with the salivary peptide diluted in PBS (20µg/mL) for 180 minutes at 37°C. Following washing and blocking steps, the blood eluates were diluted at 1:160 in PBS containing 1% of Tween20 (1%-PBST) and incubated overnight at 4°C. ELISA plates were incubated with goat anti-human biotin-conjugated IgG (Invitrogen, Thermo Scientific, USA) diluted at 1:6000 in 1%-PBST for 90 min at 37°C, followed by streptavidin horseradish peroxidase (GE Healthcare, Amersham Place, UK) diluted at 1:4000 in 1%-PBST for one hour at 37°C. The colorimetric reaction was performed using ABTS buffer (2,2'-azino-bis (3-ethylbenzthiazoline 6-sulfonic acid) di-ammonium) + 0.003% H<sub>2</sub>O<sub>2</sub> and absorbance (optical density, OD) was measured after 120 min at 405nm with a Sunrise spectrophotometer (Tecan, Switzerland).

All samples were assayed in duplicate and in a blank well (no antigen) to measure individual background and antibody response ( $\Delta OD$ ) expressed as:

$$\Delta OD = \text{mean} (OD_{Ag+}) - OD_{Ag-} \quad (1)$$

where "OD<sub>Ag+</sub>" represents the OD value in the well with the salivary antigen and "OD<sub>Ag-</sub>" the OD value in the well without the antigen.

To quantify the non-specific immune reactions and calculate the immune threshold, anti-Nterm-34kDa IgG response was assayed in individuals (n = 16) with no known exposure history to *Ae. aegypti* bites [57] (e.g., dry blood spots collected in northern France from January to March 2016 to 2018, and in Western Australia in October 2016). The specific immune threshold (TR) was defined as follows at 0.556.

$$TR = \text{mean} (\Delta OD_{\text{unexposed individuals}}) + 3 SD_{\text{unexposed individuals}} \quad (2)$$

We also defined the Mosquito Exposure Index (MEI) for each participant as

$$MEI = \Delta OD - TR. \quad (3)$$

The MEI represents the level of specific and individual IgG response to the *Aedes* salivary peptide. Individuals with a  $\Delta OD$  value above the TR, thus with a positive MEI, were classified as "immune responders" (i.e., exposed to *Aedes*). Individuals with a  $\Delta OD$  value equal or below the TR, and therefore with a null or negative MEI value, were categorized as "non-responders" (i.e., non-exposed to *Aedes*). Individuals with negative or null MEI were considered equally having a null MEI as the background immune response cannot be addressed.

### Analysis

**Covariates.** The human study population was stratified into five age groups: 5–19, 20–39, 40–59, 60–69, and  $\geq 70$  years of age. Individual's characteristics were analyzed as categorical variables to estimate their influence on the MEI. Overall travel history of each subject was used as a binary variable. At the village level, adult *Aedes* indices recorded one-month before blood collection, and immatures *Aedes* indices recorded at the time of survey were used.

Additionally, the pyriproxyfen intervention was used as a binary covariate. At the province level, the mean daily maximum and minimum air temperatures, mean percent relative humidity, and the weekly cumulative rainfall two weeks before collections were treated as covariates. The estimated 2-week time-lag takes into account potential influence on vector population biology and transmission epidemiology. Three general climatic seasons are defined according to the Thai Meteorological Department [55] with 15-February to 14-May as the hot season, 15-May to 14-October as the rainy (wet) season, and 15 October to 14-February as the cool season.

**Statistical approach.** Data analysis was conducted using R software version 3.5.1 (R Core Team, Vienna, Austria) and MASS, Rcmdr, nlme4, and lmerTest packages [58–60]. Figures were generated on R using ggplot2 and ggpvr packages [61,62]. Maps were built using QGIS software (version 3.10) and shape files were obtained from the Humanitarian Data Exchange Project [49]. As the MEI represents the specific exposition to *Ae. aegypti*, non-responder individuals were considered with a null MEI, thus the MEI was considered as a positive continuous variable (i.e.,  $MEI \geq 0$ ). The relation between MEI and entomological indices was explored using a multivariate 2-level mixed model (house, individual) with a one-month lag autoregressive correlation, assuming the antibody response persisted at detectable levels between two and six weeks [33,63]. The (1) *Aedes* adult index (2) *Aedes* adult indoor index, and (3) proportion of DENV-infected *Aedes* at the cluster level were examined in three separate analyzes. A fourth analysis was conducted with the proportion of DENV-infected *Aedes* at the household level to assess the heterogeneity of dengue transmission risk between and within study clusters. To avoid the assumption of linear relationships between antibody response to *Aedes* bites and entomological indices, risk factors were categorized into categorical variables to represent the different levels of intensity. Due to the over dispersion of mosquito numbers over time, immature stages and adult entomological indices at the cluster level were categorized into four classes, the null value of the index, and then following the terciles. The presence of DENV-infected *Aedes* was used as a binary variable (0 or  $>0$ ) due to the low number of sampled DENV-infected *Aedes*. All analyzes were performed on individuals with complete data, while individuals with missing data in covariates of interest were removed. Univariable analysis using a mixed model was conducted with each covariate to identify adjustment factors related to immune response to Nterm-34 kDa for all models. Multivariable mixed models were performed with all covariates with a  $p$ -value set at  $< 0.2$ . Subsequently, models were adjusted by backward selection and removing non-significant variables at  $p$ -value  $< 0.05$ .

## Results

### Population characteristics

The studied population, 602 individuals (318 in KK and 284 in RE), were followed-up every four months up to 19 months for an average of 3.5 visits per person (Table 1) producing a total of 3,919 collected dried blood spot samples. Among the 602 individuals recruited, a sub-sample of 92 and 71 individuals in KK and RE, respectively, were followed-up each month in sentinel sites with an average of 14.7 visits per person. The majority of the cohort was female (65.3% and 69.0% in KK and RE, respectively). The median age of the cohort was 64 and 61 years in KK and RE, respectively. The majority of the study cohort stayed most of the time at home during the weekdays and weekends (Table 2); although, in KK, about 30% of the cohort, mostly those of younger age, indicated spending some time in schools during the weekends. In KK, the vast majority of the individuals spent their weekdays indoors while in RE, about one fifth spent their weekdays both indoors and outdoors (near the location where they spend their time). Nevertheless, the behavioral trend was quite similar between KK and RE regarding daytime activities (e.g., indoor vs. outdoor locations). Most individuals were primarily

**Table 1. Population description and immunological status to Nterm-34 kDa salivary peptide.**

		Khon Kaen		Roi Et	
Population size, n individuals (no. dried blood spots)		318	(2003)	284	(1916)
Age in years, median (range of all participants)		64	(5–90)	61	(7–92)
Female proportion, % (no. females/total)		65.3	(1307/2003)	69.0	(1319/1916)
Dengue cases %, (no. cases/total)		0.00	(0/2003)	0.00	(0/1916)
Proportion of immune responder during the whole study, %, (no. responding/total)	All ages	57.3	(1148/2003)	60.0	(1150/1916)
	Age 5–19	46.7	(14/30)	53.8	(21/39)
	Age 20–39	48.9	(66/135)	64.7	(119/184)
	Age 40–59	58.9	(367/623)	60.2	(415/689)
	Age 60–69	58.2	(322/553)	54.0	(299/554)
	Age 70+	57.3	(379/662)	65.8	(296/450)

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sedentary with >95% declaring no travel in the previous 3 months before blood collections. At the time of the study, there was no evidence of incident (new) dengue infection; therefore, results presented herein is performed using entomological and immunological data only.

### Entomological collections and indices

Overall, 2,235 resting adults female *Aedes* were captured, of which the vast majority, 1,772 (79.3%) were collected indoors (Table 3). *Aedes aegypti* was the overwhelmingly predominant species identified (99.7%) compared to *Aedes albopictus* with only seven females *Ae. albopictus* collected. In Khon Kaen, 1,397 females *Aedes* (including *Ae. aegypti* and *Ae. albopictus*) were collected during a combined 1,446 house visits, the large majority (77%) captured indoors (Table 3). Moreover, DENV infection was detected among 16 females *Aedes* in KK. In Roi Et, 838 females *Aedes* were sampled from 1,441 collections, of which 696 (83%) were collected indoors. Moreover, DENV was detected among 14 females *Aedes* in RE. Additionally, 992 *Aedes* pupae (544 in KK and 448 in RE) were collected in the two cities. As with adult mosquitoes, *Ae. aegypti* pupae represented the vast majority (95.7%) of collections, therefore, all *Aedes* indices were estimated using *Ae. aegypti* and *Ae. albopictus* altogether. At the cluster level, the standard larval indices (CI<sub>c</sub>, HI, BI) indicated significantly higher *Aedes* infestation in Khon Kaen compared to Roi Et with an average of 16.4% and 4.11% *Aedes* positive containers, respectively (S2 Table). Similarly, the adult *Aedes* indices (AI<sub>c</sub> and AI<sub>in,c</sub>) were higher in KK clusters than in RE, with an average of 3.7 and 1.0 *Aedes* in KK and 0.79 and 0.68 *Aedes* in RE, respectively. Only the DENV-infected adult *Aedes* index (AI<sub>c</sub> DENV+) was higher in RE clusters than in KK with an average of 0.007 and 0.005 proportion of DENV positive *Aedes* in RE and KK, respectively. The pupal indices were, however, slightly higher in RE than in KK with 0.84 and 0.63 PHI<sub>c</sub> and 0.26 and 0.19 PPI<sub>c</sub>, respectively.

### Spatial and seasonal variation in mosquito exposure and vector density

During the study, 3,919 individual dried blood samples were collected and processed, including 2,003 and 1,916 in KK and RE, respectively. The seroprevalence rates for IgG reactivity were 57.3% and 60% in KK and RE, respectively, indicating that most individuals exhibited a specific response to the Nterm-34kDa *Ae. aegypti* salivary peptide (Table 1). The proportion of immune responders between combined RE and KK clusters was not statistically significant ( $\chi^2$  p = 0.08) (S2 Table).

In both cities, *Aedes* density (AI<sub>c</sub>) strongly increased in May-June period corresponding to the end of the hot season and the beginning of the rainy season (Fig 3). Notably, the human

**Table 2. Individual participant characteristics, behavior and occupation.** (NA: Not available).

		Khon Kaen		Roi Et	
		No. individuals = 318		No. individuals = 284	
Occupation weekdays, %, (no. answers/total)	Home	90.8	(1818/2003)	93.8	(1797/1916)
	Work away from home	7.19	(144/2003)	0.47	(9/1916)
	School/college/university	0.70	(14/2003)	0.68	(13/1916)
	Farm	1.10	(22/2003)	0.05	(1/1916)
	Other	0.10	(2/2003)	0.00	(0/1916)
	NA	0.15	(3/2003)	5.01	(96/1916)
Occupation weekends, %, (no. answers/total)	Home	69.3	(1388/2003)	94.2	(1805/1916)
	Work away from home	1.34	(27/2003)	0.05	(1/1916)
	School/college/university	29.3	(587/2003)	7.31	(14/1916)
	Farm	0.00	(0/2003)	0.05	(1/1916)
	Other	0.05	(1/2003)	0.00	(0/1916)
	NA	0.00	(0/2003)	4.96	(95/1916)
Location spent weekdays, %, (no. answers/total)	Indoor	94.6	(1895/2003)	67.4	(1291/1916)
	Outdoor	3.10	(64/2003)	0.68	(13/1916)
	Indoor and outdoor	2.00	(40/2003)	19.9	(382/1916)
	NA	0.20	(4/2003)	12.0	(230/1916)
Location spent weekends, %, (no. answers/total)	Indoor	46.0	(922/2003)	55.7	(1068/1916)
	Outdoor	0.50	(10/2003)	0.05	(1/1916)
	Indoor and outdoor	26.0	(521/2003)	18.5	(355/1916)
	NA	25.0	(550/2003)	25.7	(492/1916)
Travel in the last 14 days, %, (no. answers/total)	No	96.5	(1932/2003)	94.4	(1808/1916)
	Yes	3.54	(71/2003)	0.68	(13/1916)
	NA	0.00	(0/2003)	4.96	(95/1916)
Travel in the last 3 months, %, (no. answers/total)	No	95.3	(1909/2003)	91.6	(1756/1916)
	Yes	4.70	(94/2003)	3.390	(65/1916)
	NA	0.00	(0/2003)	4.96	(95/1916)
Travel overall during study, % (no. answers/total)	No	92.3	(1848/2003)	91.4	(1752/1916)
	Yes	7.70	(155/2003)	3.60	(69/1916)
	NA	0.00	(0/2003)	4.96	(95/1916)

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IgG response ( $\Delta$ OD) increased a few weeks after the measured peak of mosquito density. Additionally, the  $\Delta$ OD decreased from the cool season until the hot season while the mosquito densities were reduced during the rainy season with numbers rebounding during the hot season. Collectively, the results indicated a lagged positive association between *Aedes* abundance and human exposure to *Aedes* bites. Indeed, previous studies on malaria vectors showed that the time-lag for human immune response was between three- to four- weeks after the vector bites [64]. Additionally, univariate analysis of the intensity of MEI indicated a positive association between the intensity of the human Ab response and the density of adult *Aedes* collected the month before the blood spot collection (S3 Table).

### Correlations between vector infestation, vector infectivity and human exposure risk to Aedes bites

Multivariate analysis was performed on a total of 539 individuals, with complete data, including 378 individuals followed-up every four months, with an average number of 2.63 visits per person. Additionally, a sub-sample of 161 individuals, followed-up every month with an average number of 12 visits per person were included in the analysis. The models showed a strong positive correlation between the MEI and the *Aedes* adult density at the cluster level when compared

**Table 3. Entomological collection data and indices at household and cluster level.**

	Khon Kaen			Roi Et		
	Houses	Visits	Total	Houses	Visits	Total
<i>Aedes</i> female collected	179	1446	1397	179	1441	838
<i>Aedes</i> female collected indoors	179	1446	1076	179	1441	696
<i>Aedes</i> pupae collected	179	1446	544	179	1441	448
Entomological indices						
	Mean	Std dev	Range	Mean	Std dev	Range
Household level						
Adult Index DENV+	0.005	0.049	[0–1]	0.005	0.057	[0–1]
Cluster level						
Container Index <sub>c</sub> (CI <sub>c</sub> ) (%)	16.4	14.8	[0–100]	4.11	9.17	[0–66.7]
House Index <sub>c</sub> (HI <sub>c</sub> )(%)	45.5	33.8	[0–100]	12.9	21.3	[0–100]
Breteau Index <sub>c</sub> (BI <sub>c</sub> )	60.4	55.4	[0–300]	14.2	25.0	[0–137.5]
Pupae per House Index <sub>c</sub> (PHI <sub>c</sub> )	0.63	1.40	[0–10.7]	0.84	1.99	[0–10.7]
Pupae per Person Index <sub>c</sub> (PPI <sub>c</sub> )	0.19	0.45	[0–3.3]	0.26	0.72	[0–5.7]
Adult Index <sub>c</sub> (AI <sub>c</sub> )	3.71	2.42	[1–15]	0.79	0.84	[0–6]
Adult Index <sub>indoor</sub> <sub>c</sub> (AI <sub>in</sub> <sub>c</sub> )	1.00	0.87	[0–5]	0.68	0.71	[0–4]
Adult Index <sub>c</sub> DENV+	0.005	0.035	[0–0.33]	0.007	0.057	[0–0.67]

<https://doi.org/10.1371/journal.pntd.0009440.t003>

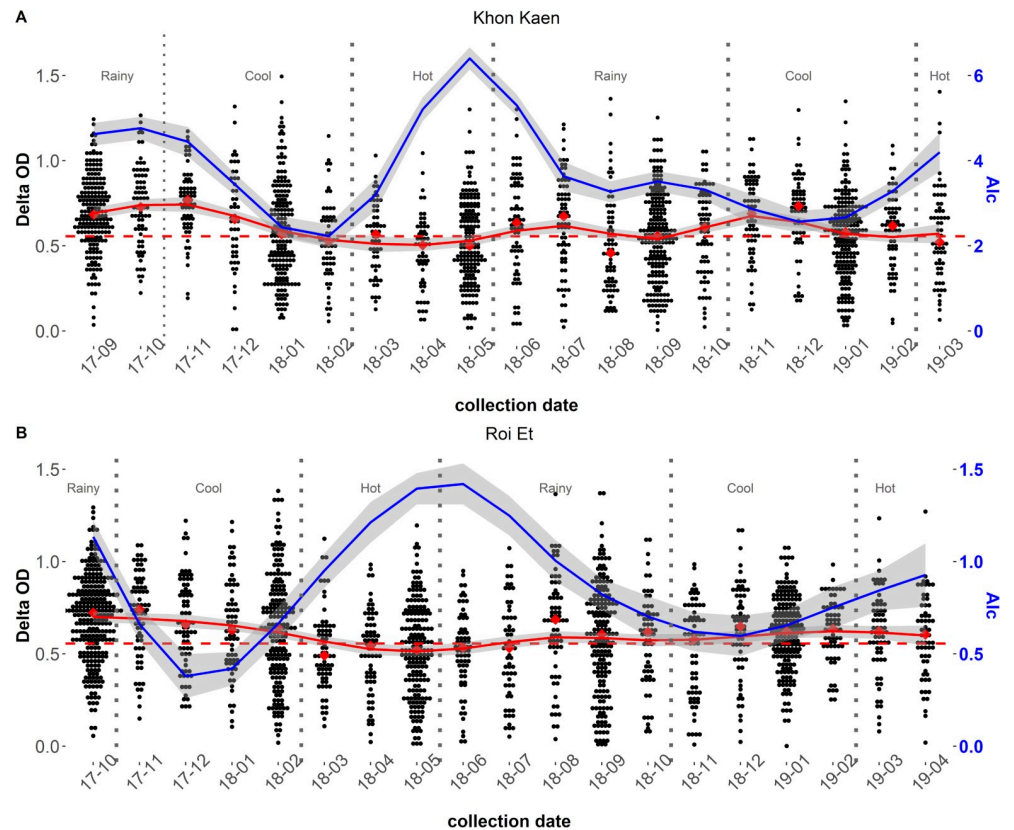
to the absence of *Aedes* for both the total adult AI<sub>c</sub> (Fig 4B and Table 4, mean difference in MEI 0.091, p<0.0001, and 0.131, p<0.0001 for medium and high level of infestation, respectively) and the adult indoor density AI<sub>in</sub><sub>c</sub> (Fig 4A and Table 4, difference in mean MEI of 0.021, p<0.007, 0.053, p<0.0001 and 0.037, p<0.0001 for low, medium and high levels of infestation, respectively). There was a significant positive association between the individual immune response and the three categories of *Aedes* intensity (low, medium and high), compared with the reference (no *Aedes*), when considering adult mosquitoes collected indoors (p<0.05).

In contrast, no clear relationships were noted between MEI and vector DENV infection at the cluster level (Table 5, p = 0.671) nor at the household level (Table 5, p = 0.764). Based on these study findings, the intensity of the immune response to *Aedes* bite exposure was not associated with a higher risk of being bitten by a DENV-infected vector (Table 5).

### Demographic, social, operational and climatic factors associated with human exposure risk to Aedes bites

For both models exploring AI<sub>c</sub> and AI<sub>in</sub><sub>c</sub>, using univariate analysis (S3 Table), all covariates (except “remain at home during the last 7 days”) were retained in the analysis. MEI differed according to age (p<0.0001), sex (p<0.0001), season (p = 0.003), vector control intervention (p<0.0001) and human occupation (p<0.0001) (Fig 4 and Table 4). The 60–69 years old age group had higher levels of antibody response to *Aedes* bites compared to other classes (Table 4 and Fig 4, p<0.001). Additionally, being male was associated with a higher risk of having had *Aedes* bites (p = 0.003 and p<0.0001) in both models. Interestingly, people spending greater time preferentially indoors during weekdays had higher levels of IgG response to salivary peptide than people spending time both indoors and outdoors (Table 4 and Fig 4, difference in MEI mean 0.036, p<0.0001 and 0.047, p<0.0001 for total *Aedes* density and indoor *Aedes* density, respectively).

Several entomological indices of immature stages were significantly correlated to the MEI. The Breteau Index was positively associated with IgG seroprevalence to the Nterm-34 kDa, although the strength of the association seemed to saturate at higher levels. Interestingly, the



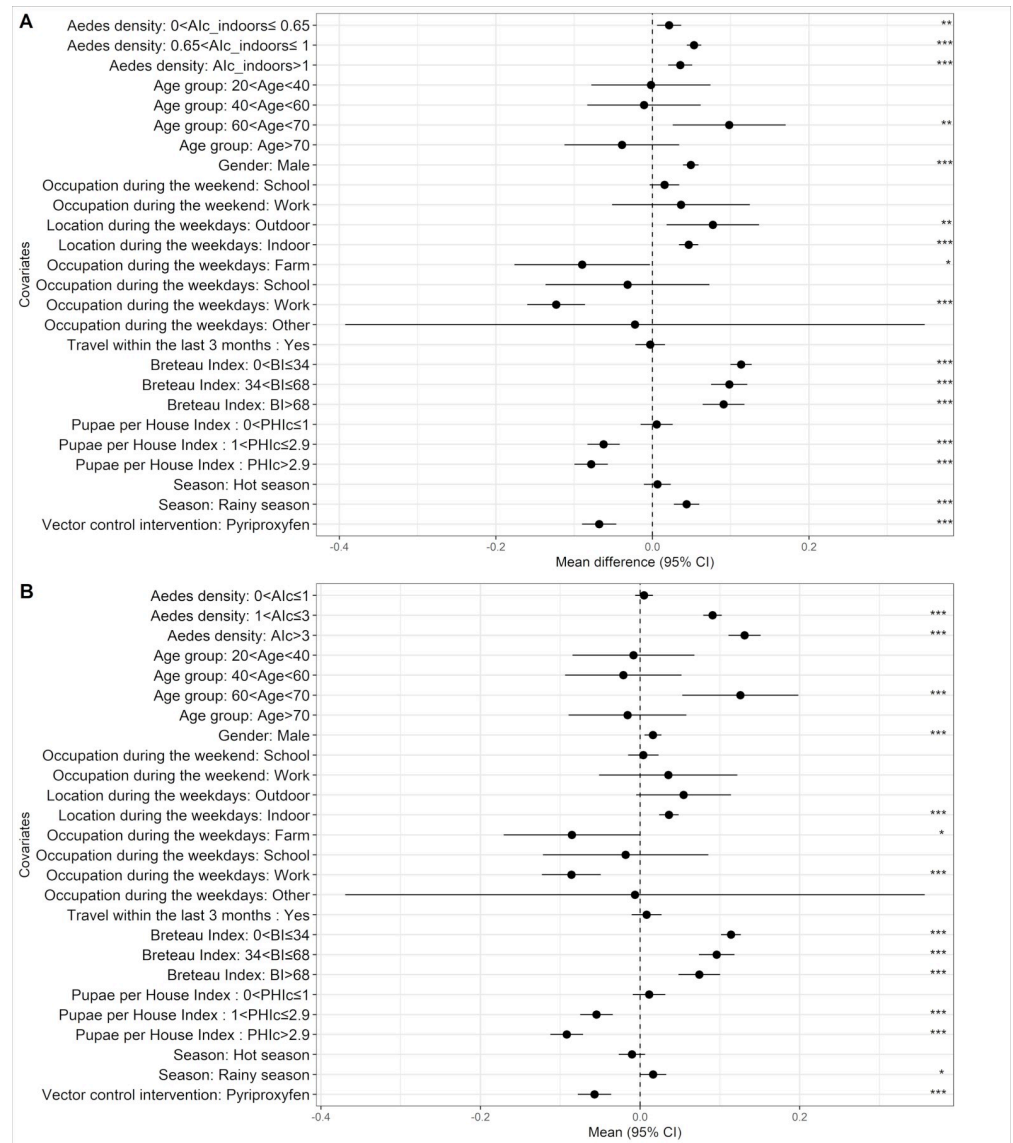
**Fig 3. Seasonal variations of the human IgG response to *Aedes* Nterm-34kDa salivary biomarker and the adult density *Aedes* Index (AI<sub>c</sub>), between September 2017 and April 2019 in Khon Kaen (A) and Roi Et (B) northeastern Thailand.** The dot plots represent the individual IgG immune response to the *Aedes* salivary peptide Nterm-34 kDa ( $\Delta$ OD). The red diamonds represent the median response during each survey. The solid red lines represent the means and the grey shaded areas represent the confidence interval of the IgG response to the salivary biomarker. The red dashed horizontal lines represent the specific immune threshold TR. The solid blue lines represent the means and the grey shaded areas represent the 95% confidence interval respectively, for the AIC at the cluster level.

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Pupae per House Index (PHI<sub>c</sub>) at the cluster level was negatively correlated with the MEI (Table 4 and Fig 4,  $p < 0.0001$ ). In both models, the presence of the trial vector control intervention was associated with a decreased level of antibody response against *Aedes* bites (Table 4 and Fig 4, difference in MEI mean -0.057 at  $p < 0.0001$  and -0.068 at  $p < 0.0001$  for the AI<sub>c</sub> and the AI<sub>in\_c</sub> models respectively). Regarding climatic factors, the rainy season was positively associated with MEI in both models.

## Discussion

This study highlights a strong positive relationship between the intensity of human IgG response against the *Aedes* salivary peptide Nterm-34kDa and adult *Aedes* population densities in association with humans in northeastern Thailand. A clear gradient response between the MEI and adult vector density indicated that individuals exhibiting higher antibody response to the *Aedes* salivary peptide were located in areas with higher risk of potential dengue vector bites. This study corroborates previous work [35–41] showing that the serological biomarker represents a promising surveillance tool to assess small-scale variations in human exposure risk to *Aedes* bites in dengue endemic settings. Although studied for malaria vectors [34], this is the first longitudinal study combining both entomological and immunological endpoints



**Fig 4. Multivariate analysis of MEI, human immune response to the Nterm-34 kDa salivary.** (A) Adult *Aedes* indoors index only multivariate model. (B) Adult *Aedes* multivariate model.

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investigating *Aedes* vectors and virus transmission. Further investigations are needed to address the kinetics of human immune response to *Aedes* salivary proteins, in particular the delay between bite exposure, and the production and waning of IgG titers.

This study showed that the human-mosquito contact is influenced by human behavioral characteristics, socio-demographic conditions, climatic factors, and trial vector control interventions associated with dengue transmission risk as previously demonstrated [8,19,56]. The relationship between human dengue infections and the intensity of the human-antibody response to *Aedes* bites could not be ascertained because incident dengue cases were not detected in the study participants during the time of longitudinal follow-up. Further analysis is on-going to confirm the observation of the apparent lack (or very low) transmission during the study period (to be reported elsewhere). In a recent case-control study conducted in northeastern Thailand (conducted by this study team), neither the adult mosquito abundance at the

Table 4. Multivariate analysis of MEI, human immune response to Nterm-34 kDa salivary peptide.

	AI <sub>c</sub> (indoors and outdoors)		AI <sub>c</sub> indoors only		
	Mean difference <sup>a</sup>	P	Mean difference <sup>a</sup>	P	
<i>Aedes</i> density <sup>c</sup>		< .0001 <sup>b</sup>		< .0001 <sup>b</sup>	
	No <i>Aedes</i>	Reference	Reference		
	Low	0.005	0.386	0.021	0.007
	Medium	<b>0.091</b>	< .0001	<b>0.053</b>	< .0001
	High	<b>0.131</b>	< .0001	<b>0.037</b>	< .0001
Age,y		< .0001 <sup>b</sup>		< .0001 <sup>b</sup>	
	Age 5–19 y	Reference	Reference		
	Age 20–39 y	-0.008	0.833	0.003	0.941
	Age 40–59 y	-0.021	0.572	-0.009	0.812
	Age 60–69 y	<b>0.126</b>	0.001	<b>0.102</b>	0.007
	Age ≥70y	-0.016	0.676	-0.039	0.313
Gender		< .0001 <sup>b</sup>		0.250 <sup>b</sup>	
	Female	Reference	Reference		
	Male	<b>0.016</b>	0.003	<b>0.048</b>	< .0001
Occupation during the weekdays			0.002 <sup>b</sup>	0.028 <sup>b</sup>	
	Home	Reference	Reference		
	Work away from home	<b>-0.086</b>	< .0001	<b>-0.124</b>	< .0001
	School	-0.018	0.732	-0.031	0.568
	Farm	<b>-0.086</b>	0.050	<b>-0.088</b>	0.049
	Other	-0.006	0.972	-0.018	0.923
Occupation during the weekends		< .0001 <sup>b</sup>		< .0001 <sup>b</sup>	
	Home	Reference	Reference		
	School	0.004	0.685	0.015	0.128
	Work	0.035	0.425	0.035	0.446
Location during weekdays		< .0001 <sup>b</sup>		< .0001 <sup>b</sup>	
	Indoors and outdoors	Reference	Reference		
	Indoors	<b>0.036</b>	< .0001	<b>0.047</b>	< .0001
	Outdoors	0.055	0.0723	<b>0.077</b>	<b>0.012</b>
Travel overall		< .0001 <sup>b</sup>		< .0001 <sup>b</sup>	
	No	Reference	Reference		
	Yes	0.008	0.393	-0.004	0.657
Breteau Index		< .0001 <sup>b</sup>		< .0001 <sup>b</sup>	
	0	Reference	Reference		
	0–34	<b>0.114</b>	< .0001	<b>0.114</b>	< .0001
	34–68	<b>0.096</b>	< .0001	<b>0.098</b>	< .0001
	>68	<b>0.074</b>	< .0001	<b>0.091</b>	< .0001
PHI <sub>c</sub>		< .0001 <sup>b</sup>		< .0001 <sup>b</sup>	
	0	Reference	Reference		
	0–1	0.011	0.281	0.006	0.689
	1–2.9	<b>-0.055</b>	< .0001	<b>-0.060</b>	< .0001
	>2.9	<b>-0.092</b>	< .0001	<b>-0.078</b>	< .0001
Season			0.003 <sup>b</sup>	< .0001 <sup>b</sup>	
	Cool	Reference	Reference		
	Hot	-0.010	0.2258	0.006	0.474
	Rainy	0.016	0.054	<b>0.044</b>	< .0001

(Continued)



Table 4. (Continued)

	AI <sub>c</sub> (indoors and outdoors)		AI <sub>c</sub> indoors only	
	Mean difference <sup>a</sup>	P	Mean difference <sup>a</sup>	P
<b>Vector control intervention</b>		< .0001 <sup>b</sup>		< .0001 <sup>b</sup>
Control	<b>Reference</b>		<b>Reference</b>	
Pyriproxyfen	<b>-0.057</b>	< .0001	<b>-0.068</b>	< .0001

Analyses adjusted for rain, temperature maximum and cluster variables, in addition to the other specified variables. The difference in mean MEI in bold is significant at 0.05.

<sup>a</sup> Defined as the difference between each class and the reference category

<sup>b</sup> Likelihood ratio test to assess the global effect of the variable.

<sup>c</sup> Adult density categories: 0–1, 1.1–3, and > 3 for AI<sub>c</sub> class, low, medium and high, respectively. 0–0.65, 0.66–1 and >1 for AI<sub>in,c</sub> *Aedes* female collected indoors categories low, medium and high, respectively.

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household level nor the degree of human exposure to *Aedes* bites was correlated with a higher odds of acquiring dengue infection [56]. Although consistent with some previous results in Southeast Asia [43,56], the small sample size of DENV-positive *Aedes* might explain the lack of significance between the human-*Aedes* exposure and the risk of DENV infected bites seen in this study. This highlights dengue virus transmission is both a multi-factorial and a complex affair that varies over time and space, and the relationship between vector density and virus transmission is dynamic and thus might not be adequately or accurately characterized through standard methods of entomological monitoring.

These findings show that the MEI was significantly associated with the season and prevailing climatic factors. The proportion of immune responders to *Aedes* bites was higher during the rainy season than the drier months of the year, corresponding to the period of greater adult vector densities. This is probably explained by the dramatic increase in most entomological indices during this period of the year where the number of suitable larval habitats increases and adult survival (longevity) is presumably enhanced [15,65]. Similar results were reported in Benin, where the overall anti-saliva antibody response in children increased during the rainy season [42]. A recent study in Cote d’Ivoire highlighted a strong relationship between human mosquito exposure, season and agricultural practices [66]. Specific IgG responses remained high during both seasons in villages associated with intensive agricultural compared to villages lacking agricultural practices. The authors suggest that the presence of rubber and oil palm plantations, by providing a suitable environment for the presence of *Aedes* vector species maintained a high level of human exposure to *Aedes* mosquito bites regardless of annual seasonal changes.

Table 5. Multivariate mixed linear model of human immune response to Nterm-34kDa *Aedes* salivary peptide or MEI and the presence of DENV infected *Aedes* in the cluster.

	Cluster level		Household level	
	Mean difference <sup>a</sup>	P	Mean difference <sup>a</sup>	P
<b>DENV infected <i>Aedes</i></b>		0.003 <sup>b</sup>		0.050 <sup>b</sup>
0	<b>Reference</b>		<b>Reference</b>	
> 0	0.012	0.671	-0.015	0.764

Analyses were adjusted for age, gender, travel history, BI<sub>c</sub>, PHI<sub>c</sub>, season, and cluster variables, in addition to the other specified variables. The difference in mean MEI immune response in bold are significant at 0.05.

<sup>a</sup> Defined as the difference between each class and the reference categories.

<sup>b</sup> Likelihood ratio test to assess the global effect of the variable.

<https://doi.org/10.1371/journal.pntd.0009440.t005>

Interestingly, the present study also suggests correlations between the MEI and *Aedes* immature-based indices, although the association appeared weaker compared to adult measures. The Breteau Index was associated with higher levels of antibody response against *Aedes* bites but was not gradient-dependent. In contrast, the pupae per house index was negatively associated with the MEI. This result might seem contradictory; however, that under natural field conditions, larvae and pupae development rates are strongly influenced by climatic factors, particularly ambient temperature and rainfall patterns, as well as density-dependent factors of immature stages affecting resource competition [67–69]. Additionally, the presence of larval stages in an aquatic habitat can inhibit further egg hatching [70]. Therefore, a decrease in human immune response to *Aedes* bites could be the reflection of the cyclic fluctuations between successive adult population densities influenced by site-specific immature mosquito densities.

The MEI varied according to individual characteristics, such as gender, age, and occupation. Interestingly, older people presented higher risk for mosquito bites than the younger population. Similarly, being a male was associated with a higher exposure level to *Aedes* bites. Similar results were found with *Anopheles* exposure and malaria transmission in Thailand, where males were at higher risk than females, mainly due to differences in behavior and occupational exposure [37]. Nevertheless, these results have to be viewed with caution as the majority of the participants in the present study were female and the median age of the cohort was 64 in KK and 61 in RE, which may have biased the outcomes. Indeed, the median age of the cohort reflects the lack of representation of the younger population, which are presumed more active (mobile) than older individuals. Our findings also showed that individuals spending the majority of time indoors were associated with a higher exposure to *Aedes* bites than those spending time more equally either indoors and out. An explanation is that *Ae. aegypti* is a well-adapted species for resting and breeding inside dwellings, and is more typically found indoors [22,23]. This is also supported by the level of significance of human-exposure risk using the *Aedes* indoor index. The risk of biting (i.e., transmission) inside a dwelling appears particularly important and helps explain why insecticide-treated curtains and targeted indoor residual spraying were highly effective against *Ae. aegypti* for the control and prevention of dengue outbreaks in Mexico and Australia [46,71].

This study suggests that the salivary biomarker is sensitive enough to detect small scale variations in human exposure to *Aedes* bites over time, in particular during a vector control intervention. The human IgG levels were significantly lower in treated clusters compared to the control clusters. These findings would suggest an appreciable impact of pyriproxyfen treatment on the density of *Aedes* adult populations. Similar results were observed in La Réunion, where vector control intervention combining *Aedes* larval habitat source reduction and insecticide space spray against adult mosquitoes was associated with a significant decrease in human antibody response against *Ae. albopictus* bites [41,63]. Investigations are on-going in Thailand to assess the entomological and epidemiological impact of pyriproxyfen intervention in the study area [48,72].

This study represents an important step toward the validation of using the *Aedes* salivary peptide Nterm-34kDa as a proxy measure to assess *Aedes* infestation levels and human-mosquito exposure risk in a dengue endemic area. Although promising results are described, the use of the Nterm-34 kDa as a surveillance indicator for estimating dengue transmission risk requires further investigations including other geographical and transmission settings.

## Supporting information

### S1 Table. Defined variables.

(DOCX)

**S2 Table. Comparison of proportion of immune responders and entomological indices between Khon Kaen and Roi Et provinces using Chi square test and ANOVA.**

(DOCX)

**S3 Table. Univariate analysis of the human antibody response to the Aedes salivary biomarker Nterm-34 kDa.**

(DOCX)

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**Writing – original draft:** Benedicte Fustec, Vincent Corbel.

**Writing – review & editing:** Benedicte Fustec, Michael J. Bangs, Neal Alexander, Hans J. Overgaard, Vincent Corbel.

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