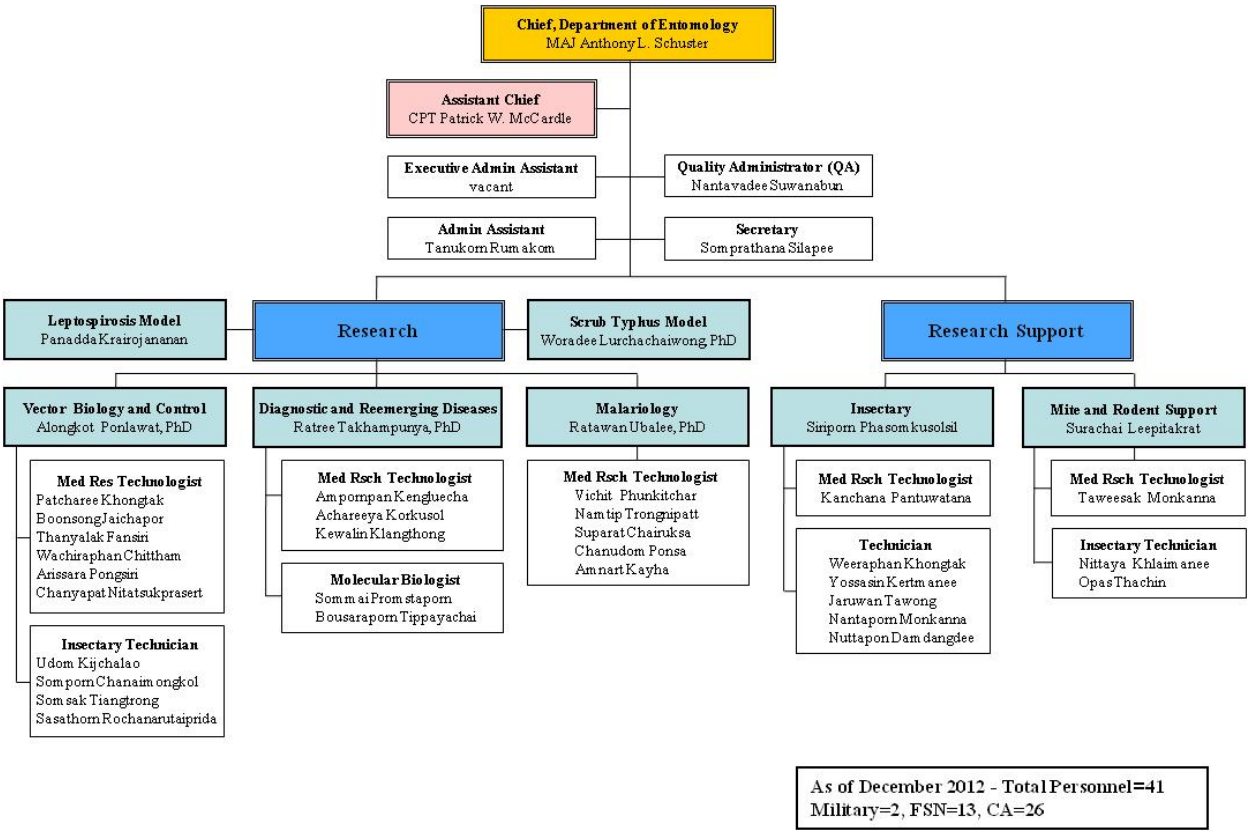
DEPARTMENT OF ENTOMOLOGY

DEPARTMENT MISSION

To **identify** vector- and rodent-borne disease threats of military and global health importance and to **develop and evaluate interventions and products** in order to mitigate those threats.

PERSONNEL



IN-HOUSE TRAINING PROGRAMS AND OUTSIDE TRAINING OF PERSONNEL

In-House Training Programs

- Accident, Illness and Complaint Reporting, Records and Investigations
- Animal Bites and Other Injuries/Reports
- Animal BSL-3 Hazard Training
- B-Virus Post-Exposure Intervention and Prophylaxis
- Bio-Safety in Laboratory
- Blood-Borne Pathogens Exposure Control Plan
- BSL-3 Laboratory Training Program
- Computer Security Awareness
- Composite Risk Management
- Chemical Safety

- Counter Intelligence, Operations Security, and Anti-Terrorism
- Equal Employment Opportunity and Prevention of Sexual Harassment (EEO & POSH)
- Ethics Training
- Fire Prevention, Protection, Report and Investigation
- Fire Fighting Training
- Human Subject Protection Program
- The Humane Care and Use of Lab Animals
- Hazard Communication Program
- IACUC Training
- Laboratory Waste Management
- Occupational Health Program & Communication
- Post-Exposure Prophylaxis and Interventions
- Respiratory Protection Program
- Routine Prophylaxis and Screening and Post-Exposure Prophylaxis and Intervention
- Staff Training on Command SOPs, Protocols, Departmental SOPs
- Safety Equipment Usage
- Basic Supervisory Skills Workshop, Foreign Service Institute U.S. Department of State
- Regional Training/Workshop on Rodent and Rodent Borne Pathogen Surveillance: from Trapping to Pathogen Screening, Kasetsart University
- Morphological Identification of Medically Important Chiggers, Centers for Diseases Control (CDC), Taiwan
- Laboratory Training on Basic Molecular Techniques for Ms. Weerawan Charnsilpa, Faculty of Medical Technology, Hauchiew Chalermprakiet University, Samut Prakarn
- Hosted a Master of Science student from University of Montpelier, France. His Research Project was entitled "The molecular cophylogenetic relationship between larval trombiculid mites and their rodent hosts in Southeast Asia: implications for the maintenance and transmission of scrub typhus"
- Two students from Thai University for 90 days VBC
- U.S. State Department Summer Hire Program, one student Insectary, one student DRD
- Laboratory Techniques in Mosquito Infection, Mosquito Dissection, Evaluation of Oocyst and Sporozoites from Plasmodium Infected Mosquitoes for Transmission Blocking Drug Activity Assay to ten employees of RCF/CNM, Anlong Veng, Cambodia, and 7 staff members of AFRIMS Department of Immunology

Outside Training Provided by Department

- Dengue Prevention Consortium, Ministry of Public Health, Bangkok, Thailand
- Malaria Vector Identification Workshop, Ministry of Public Health, Surat Thani, Thailand
- Mosquito Surveillance and Identification Workshop, Siem Reap, Cambodia
- TALAS Annual Training
- Dengue and Malaria Vector Control Workshop, Ministry of Public Health, Rayong
- Mosquito Biology and Dengue Vector Control Workshop, Ministry of Public Health, Kamphaeng Phet

RESEARCH AREAS

Vector Biology and Control: Develops and evaluates mosquito surveillance traps; evaluates control or preventive measures including insecticide barrier treatments, insecticide-impregnated bed nets and tents, and personal-use and spatial repellents. Studies are aimed at

understanding basic vector biology and behavior through vector competence and ovi position assays. Taxonomic keys are continually developed and updated for the mosquitoes of Thailand. Apply Geographic Information Systems (GIS)-based tools to evaluate the risk of vector-borne disease outbreaks. This capability incorporates environmental factors and field collection data of vector/pathogen distributions in order to delineate vector-borne disease such as dengue, Chikungunya and Japanese encephalitis viruses, and malaria risk areas.

Diagnostic and Reemerging Diseases: Develops and evaluates conventional and rapid field assays for the detection of emerging/re-emerging pathogens. Conduct cutting edge bio-informatics and molecular research on arthropod-borne disease vectors (molecular identification of mites) and pathogens (*Leptospira*, *Orientia*, *Bartonella, Plasmodium*, Chikungunya and dengue viruses), and the relationships among the pathogen, vector reservoir, and host.

Malariology: Conducts research on malaria biology and transmission. On-going research includes studies on malaria parasite development in mosquitoes, erythrocyte and liver cells. Develop *in vitro* systems for the screening of antimalarial compounds against exoerythro-cytic, gametocytic and sporogonic stages of malaria parasites. Studies are also focused on gametocytogenesis in *Plasmodium falciparum* and the production of different stages of human and animal malaria parasites in order to support drug and vaccine development, and transmission blocking strategies.

Leptospirosis Model: Conducts research on leptospirosis pathogen biology and host-vector relationships. Evaluate environmental factors that affect transmission and maintenance of pathogen across diverse geographical differences. Correlate vector, reservoir, and host genotypes across divergent geographical areas with a focus on phylogenetic relationships.

Scrub Typhus Model: Conducts research on scrub typhus epidemiology within vectors, reservoirs and hosts. Establish and maintain colonies of *Leptotrombidium* mites to conduct vector competency and vaccine challenge studies. Develop repeatable standardized chigger-mouse model to further investigate transmission studies. Develop chigger-nonhuman primate model to more closely mimic human reactions to chigger feeding and pathogen transmission in order to support drug and vaccine development and transmission blocking strategies.

Insectary: Produce world class superior quality standard mosquitoes for malaria, dengue, and Japanese Encephalitis research. Rears and researches biology of 5 endemic Thai malaria vectors for transmission studies in support of internal Veterinary Medicine, Entomology and Immunology transmission blocking, vaccine, and prophylactic compound challenges.

Mite and Rodent: Maintain endemic scrub typhus chigger colonies for biological studies and ongoing transmission studies. Provide infected and non-infected chigger mites in support of NHP model and mouse model development for scrub typhus vaccine evaluations. Additionally, supports *Orientia tsutsugamushi* transmission and immunological investigations involving rodents and mites. Collects and identifies rodents/mites in support of rodent-borne disease surveillance and transmission studies.

ACCOMPLISHMENTS

1. **Title: Field Evaluation of Surveillance Traps for Malaria Vectors**
   Funding Source: MIDRP U

Project Summary: The overall goal of this research was to evaluate the efficacy of commercially-available mosquito traps for potential use by military preventive medicine personnel as a component of comprehensive mosquito surveillance and control program. The specific aims/hypotheses in the proposed research were: 1) to evaluate the efficacy of Centers for Disease Control and Prevention Light Traps (CDC Light Trap) and modified CDC Light Traps (a BG-sentinel fan was attached below the CDC Light Trap collection net) using different

attractive sugar baits (ASB) for the collection of malaria vectors in Thailand, and 2) to evaluate the efficacy of ASB compared with a control (cotton ball soaked with water) against, *An. dirus* and *An. minimus*. The ASB solution consisted of combination of 30% guava juice, 30% pine-apple juice, 26% water, 12% sugar, and 2% fermented sweet rice. The semi-field experimental studies were carried out in large mosquito enclosures (LME) located in Kamphaeng Phet Province, Thailand.

Results showed that the CDC Light Trap baited with BG-Lure/CO2/ASB2 (fermented sweet rice) had high potential for malaria vector surveillance and can be effectively used as the new gold standard technique to collect malaria vectors in Thailand. The modified CDC Light Trap (CDC Trap + BG fan) baited with BG-Lure and CO2 together was more efficient in collecting malaria vectors. The data will aid the researcher select the most effective and appropriate ASB for combination with CDC Light Traps to increase the efficiency of sampling devices in this region.

CY13 Plan: We will evaluate the efficacy of the CDC Light Trap and the modified CDC Light Trap with combinations of different baits to include BG-Lure/CO2/ASB2 (fermented sweet rice) under field conditions. Data will be analyzed and published.

2. **Title: Dengue Vaccine and Viral Evolution in Rural Thailand**

Funding Source: NIH RO1 (Multi-center collaboration)

Project Summary: We collected adult mosquitoes within a 200 m radius of patients infected with dengue during a cluster investigation to determine if genetic diversity of dengue viruses and the vector mosquitoes change with exposure to vaccines. In 2012, 123 clusters were investigated (more than 10,000 houses in 34 villages have been mapped). Immature mosquitoes were collected from the field and transported to the AFRIMS Entomology Lab, where they were allowed to emerge as adults. Genetic diversity among adult mosquitoes and vector competence was assessed at the AFRIMS lab. The specific aim will be determined by comparing results from dengue virus genetic analyses and vector competence assays to the genetic identity of mosquitoes. A minimum of two DENV serotypes will be studied; specific serotypes will depend on those most frequently recovered in cluster investigations. Molecular analysis is ongoing. These activities are to support a larger effort to determine the evolution of the dengue virus in response to a vaccine.

CY13 Plan: Research efforts will continue thru the next three years. Data will be analyzed and published.

3. **Title: Population Densities and Malaria Infection Rates for *Anopheles* Species in a Malaria Endemic Area of Cambodia**

Funding Source: Global Emerging Infections Surveillance and Response System (GEIS)

Project Summary: The goals of this study were to:

1. Determine the relative abundance and distribution of *Anopheles* spp. using CO_2-baited light traps in select habitats; e.g., forested (military communities) and deforested (civilian inhabited) areas.
2. Confirm identification of *Anopheles* spp. by PCR for members of selected species complexes when morphological identification is not reliable.
3. Determine and compare the sporozoite (head/thorax) infection rates of *P. vivax*, *P. falciparum*, and mixed *Plasmodium* spp. infections for each *Anopheles* spp.
4. Identify seasonal trends incorporating human disease data (local health clinics and hospital data, Ministry of Health data) with associated mosquito species/infection data.

The purpose of the survey was to collect a maximum number of *Anopheles* spp. to identify relative species abundance and seasonal and habitat distribution. Therefore, CDC Light Traps were placed on the periphery of the village between *Anopheles* spp. breeding sites and human populations.

CY13 Plan: The project will continue. Mosquitoes will be collected over a 12-h period (1800 to 0600 hrs) for four consecutive nights per month over a 12-month period from approved collection sites in rural areas of Cambodia along the Thai border.

4. Title: Genetic Basis of Natural Compatibility between Dengue Viruses and Mosquito Vectors

Funding Source: French National Research Agency (ANR), France

Project Summary: The project constituted a complementary approach to an existing collaborative project involving several research groups in the U.S.A. and in Thailand. We wanted to gain a better understanding of vector-mediated constraints to arboviral emergence and evolution by investigating the genetic basis of natural vector-virus interactions between *Ae. aegypti* and dengue viruses.

CY13 Plan: Analyze data for publication.

5. Title: Development and Evaluation of Novel Push-Pull Strategies for the Control of Disease Relevant Mosquito Species

Funding Source: MIDRP U

Project Summary:

1. Outdoor mosquito enclosures: Three large mosquito enclosures (LME) (16.8 m long x 12 m wide x 5.5 m high) were constructed in a dengue-endemic area at Nongpling sub-district, Muang district, Kamphaeng Phet Province (KPP) (Fig. 1). A water drainage system was installed and connected to the enclosures. Each LME is covered with polyester netting thereby allowing the free entry of both wind and precipitation and creating climatic conditions within the enclosures that mimic ambient conditions. The roof of each LME is covered with thick white polyethylene plastic to afford some degree of protection from intense seasonal rains. Entrance into the large screened cage is through a double layer door system to prevent escape of released mosquitoes from within the cage.

Figure 1 Large Mosquito Enclosures (LME) (16.8 x 12.0 x 5.5 m) Constructed in Nongpling Subdistrict, Muang District, Kamphaeng Phet Province, Thailand

2. Outdoor mosquito enclosure calibration: Calibrations were conducted in order to demonstrate that mosquitoes show relatively equal preference for the same trap model located at 4 corners of each LME. Procedures were performed using commercially-available surveillance traps that have shown efficacy in collecting the mosquitoes species used in our study. BG-Sentinel traps with BG-Lure and CO_2 were used and operated according to the manufacturer's instructions to collect *Ae. aegypti*, while CDC Light Traps baited with dry ice were used for *An. minimus* collection. Each LME was divided into four equal sections (each 8.4 x 6 m). Four traps of the same type were randomly placed at the center of each section. Two hundred host seeking females (4-7 day-old) and two hundred males were released into the cage at its midpoint. *Aedes aegypti* were released for 3 hrs from 7-10 am, while *An. minimus* were released for 13 hrs from 6 pm-7 am. Traps were switched on before mosquitoes were released at the midpoint of the LME. Four replicates were performed with each trap being rotated clockwise after each replicate. Collected mosquitoes were sorted by sex and counted. Each LME was voided of mosquitoes after each replicate using traps and CDC backpack aspirators.

3. Indoor and outdoor preferences of mosquitoes within LMEs: Both vector species used in this study are different in their host seeking behavior. Female *Ae. aegypti* seek their hosts during the day time. They also prefer resting inside of structures where humans dwell whereas *An. minimus* normally live outside and seek human or animal hosts during the night. An experiment was performed to determine whether semi-field conditions within LMEs affect the normal behavior of these two mosquito species. Grass and potted foliage plants were grown in each LME to simulate the outdoor environment inside the LME. A PVC-coated polyester tent (3.0 x 3.0 x 2.10 m with its roof 2.65 at the apex) with a mesh-net door and 3 windows were positioned 3 meters from the center of the each LME (Fig. 2) to mimic a human dwelling. BG-sentinel traps with BG-lure and CO_2 and CDC Light Traps with CO_2 were used in this experiment. *Ae.aegypti* were collected in the morning at 0600-0700 hrs, while *An. minimus* collection was performed at night time, 1900-2000 hrs. One trap was placed inside the tent and the other one was placed outside the tent (6 m from the tent). A plastic cup containing 400 mosquitoes (200 males, 200 females) was placed at the midpoint of the LME. Each mosquito cup was remotely opened after traps had already become operational.

Figure 2 A PVC-Coated Polyester Tent (3 x 3 x 2.1 m) with Mesh-Net Door and 3 Windows (60 x 60 cm) Placed Inside Each LME.

4. Attractive bait "Pull" evaluations. Three candidate "Pull" devices including Pro-Vector®Entobac® (MEVLABS, Inc. U.S.A.), dry ice, and BG-Lure were evaluated against *Ae. aegypti*. For *An. minimus*, Netty®with EntobacD® (MEVLABS, Inc. U.S.A.), dry ice, and the BG-Lure were tested. ProVector® Entobac® is a new product that consists of a durable plastic flower with a replacement bait pad containing a new formulation of *Bacillus thuringiensis israelensis* (Bti). The Netty® (MEVLABS, Inc, U.S.A.) device is a bait-pesticide card that consists of a piece of paper with a color pattern on one side and EntobacD pesticide (a combination of Bti and deltamethrin) on the opposite side that can rapidly kill mosquitoes. Pull device evaluations using cubic sticky traps were carried out under non-human bait conditions.

5. Evaluation of the "Pull" devices versus the "Push-Pull" devices. BG-sentinel traps baited with BG-lure and CO_2, and a CDC Light Trap baited with CO_2 and BG-lure were used as the "Pull" devices for *Ae. aegypti* and *An. minimus* collection, respectively. A metofluthrin impregnated net (MIN), SumiOne® (Sumitomo Chemical, Japan) was evaluated in this experiment as the "Push" device. We evaluated the efficacy of the "Pull" devices and the "Push-Pull" devices, and compared them with the control (without devices) against *Ae. aegypti* and *An. minimus*. One hundred host-seeking females (4-7 d-old) were remotely released from the mid-point of each LME. *Ae. aegypti* mosquitoes were released for 2 hrs (0700-0900) while *An. minimus* mosquitoes were released for 13 hrs (1730-0630 hrs). Doors and windows of the tents remained opened throughout these periods. After the appropriate time period, mosquitoes inside the tent in each LME were captured and counted. Devices were rotated counterclockwise after each collection time which comprised one replicate of the study. Six replicates for each mosquito species were conducted.

6. Evaluation of the "Push" devices versus the "Push-Pull" devices. We evaluated the efficacy of the "Push" devices and the "Push-Pull" devices, the reverse of the above study, as compared with the control (without devices) against *Ae. aegypti* and *An. minimus*.

CY13 Plan: Two 50-m tunnels will be constructed in Chanthaburi Province. New attractants and spatial repellents will be tested in 50-m tunnels under field conditions.

6. Title: Evaluation of a Tent Trap for Collection and Study of Disease-Carrying Mosquitoes

Funding Source: WRAIR

Project Summary: The objective of this investigation is to evaluate the effectiveness of SkySight Technologies prototype Tent Trap for the collection and study of disease-carrying blood feeding flying insects (primarily mosquitoes) compared to the currently available CDC-Standard CO_2 Trap. Test of trap effectiveness will be conducted in real-world open-air conditions (full field) at Kaeng Hang Maeo, Chantaburi Province, Thailand.

The test series will consist of 12 nights of testing: Each night, three Tent Traps will be tested: two prototype SkySight Tent Traps (each using one person inside the tent as the attractant) and one CDC-standard trap (using a CO_2 canister as the attractant). The three sites will be at situated at least 50 meters from one another. After each night of testing, the three traps will be rotated among the three; thus over the 12-night test series, each Tent Trap will be at any given site for four nights, eliminating any site-specific influences in the data.

The test site has been established at Kaeng Hang Maeo, Chantaburi Province, Thailand. This site has been used in previous studies due to the abundance of surrounding mosquito habitat and location security. Testing will occur within a walled compound enclosing sufficient area to maintain proper distances between traps while preventing intrusion by passers-by. Two such 12-night test series schedules are planned resulting in 72 total trap nights (3 traps X 12 nights X 2 test periods).

CY13 Plan: Experiments will begin June 2013.

7. Title: Development of a DNA-Based Assay for the Detection of Human Malaria Parasite Species: Proof of Concept for an Oligochromatographic Format

Funding Source: MIDRP L

Project Summary: The commercially-available rapid test currently in use is largely based on the antigen-antibody capture system. DNA-based detection assay would be a complementary/confirmatory test to the existing rapid test. LAMP are routinely performed in our laboratory and evaluated relative to conventional microscopic examination. The test will be designed to detect parasites in the genus *Plasmodium* that include *P. vivax*, *P. falciparum*, *P. ovalae*, *P. malariae,* and *P. knowlesi*. Malaria genus-specific and species-specific (*P. vivax*, *P. falciparum*) LAMP primers were designed and validated. The sensitivity and specificity of our primers were comparable with or better than that of previously published primers (Han *et al*., 2007). The DNA extraction method was optimized to reduce the time and steps to less than 20 minutes which is suitable for field use.

Our initial objective was to develop a DNA-based malaria diagnostic test with oligochromatographic format using the basic principle of Loop-mediated DNA amplification. We have adjusted this project objective so the product of this project can be transitioned to our continuing project (L0314_13_WR_PP_OC: Lyophilized, Thermo-Stable LAMP Chemistries for the detection of the Malaria, Dengue, and Chikungunya).

CY13 Plan: These primer sets will be further used in freeze-dry chemistry development (L0314_13_WR_PP_OC: Lyophilized, Thermo-Stable LAMP Chemistries for the detection of the Malaria, Dengue, and Chikungunya).

8. Title: Rodent-Borne Surveillance in Thailand Focusing on Leptospirosis, Scrub Typhus, and Rickettsial Disease

Funding Source: GEIS sustainment

Project Summary: To better understand the epidemiology of leptospirosis, and scrub typhus we conducted systematic sampling of rodents from provinces in the North, Central, and Northeast during both dry and wet seasons. Eight provinces were included in this study: Nan/Tak (North), Rayong/Trat (East), Sing Buri/Phra Nakorn Sri Ayutthaya (Central), and Kalasin/Burirum/Nakhon Ratchasima/Sisaket (Northeast). Rodent tissues and their associated ectoparasites were screened for the presence of leptospirosis, scrub typhus, and rickettsial disease. We collected 562 rodents which were separated into 206 pools of ectoparasites (chiggers, fleas, and ticks). Of the 768 samples tested, 15 were positive for *O. tsutsugamushi* (1.95%), 24 positives for pathogenic leptospira (3.12%), and 8 positives for *Rickettsia* (1.04%). Two leptospira species previously reported as the predominant species in Thailand, *L. interrogans* and *L. borgpetersenni*, were detected from samples in this study. The majority of *O. tsutsugamushi*-infected samples are Kato genotype; however, TA678 and Gilliam were also detected. Five out of eight *Rickettsia* were identified into typhus group (*R. typhi*), while the remaining three *Rickettsia* belong to spotted fever group (SFG). Analysis is ongoing and will be published.

CY13 Plan: Extend current rodent-borne disease surveillance into other regions of Southeast Asia (Thailand, Laos, and Philippines)

9. Title: Development of a Molecular Taxonomic Key for the Identification of Medically-Relevant Trombiculid Species from Korea, Japan, and Thailand

Funding Source: MIDRP U (FY12-13)

Project Summary: Develop a molecular taxonomic key to identify chigger specimens collected from Thailand and other countries (Korea, Laos, Cambodia). Each morphologically-identified species can be associated with a DNA barcode (COI gene, 12-16S rDNA, and ITS2). Current morphological keys for mites are not as accurate as necessary to properly identify/

differentiate vector and non-vector mite species by anyone but the most experienced taxonomist. We are developing molecular keys for mites using field-collected specimens within the trombiculid family (not only *Leptotrombidium*). Specimens are identified morphologically and undergo DNA extraction/amplification. Sequence and phylogenetic analysis will be performed using all genes mentioned above. This molecular taxonomic key will be used to enhance the capability at AFRIMS for morphological identification of mite specimens from South East Asia. Currently, we are able to discriminate the trombiculidae mites (subfamily level) into tribe, genus, and subgenus especially for *Leptotrombidium* mites. Additional key morphological characteristics used for species identification of *Leptotrombidium* (*Leptotrombidium*) will be recorded for each specimen. The association between morphology and DNA sequences will be determined from our data. Nineteen chiggers were successfully reared in our lab and their F1 progenies were divided into two groups; one was subjected to DNA extraction and gene sequencing, another was slide-mounted and used for morphological ID. Thirteen slides were sent to the Walter Reed Biosytematics Unit for morphological identification by their mite taxonomist.

CY13 Plan: Incorporate the DNA barcode with morphological characteristics of chiggers

10. Title: Detection of *Plasmodium vivax* Pre-Erythrocytic Stage Using a Rolling Circle Amplification (RCA) Assay

Funding Source: ILIR (FY10-12)

Project Summary: We established *in situ* RCA assays for malaria parasite detection in liver cells. The assay was applied to *P. vivax*-infected HepG2-A16 cells (liver cells). Freshly prepared *P. vivax* sporozoites (infective stage) harvested from *Anopheles dirus* that fed on *P. vivax*-infected patient blood were used in the invasion experiment. *P. vivax* sporozoites (7.5×10^5) were added per well to a HepG2-A16 cell monolayer and maintained in 5% CO_2, 37°C incubator. The invasion of *P. vivax* was detected by RCA technique in comparison with IFA staining using HSP70 antibody. *P. vivax* invasion of hepatic cells was successfully detected by RCA technique using a universal probe or *P. vivax*-specific probe. The positive signal was confirmed by IFA staining using HSP70 antibody. The successful *in situ* RCA condition will be applied to our future research involving characterization of liver-stage *P. vivax* development or hypnozoite formation. Knowledge on liver-stage *P. vivax* biology will greatly advance vivax malaria research community.

CY13 Plan: N/A

11. Title: Performance of *Plasmodium falciparum* (Pf) and *P. vivax* (Pv) Transmission Blocking Assays in Support of Transmission Blocking Vaccine Studies

Funding Source: NIH

Project Summary: Malaria caused by *Plasmodium* spp. is one of the most important parasitic diseases of mankind. A novel approach to the control of malaria is the use of transmission blocking vaccine (TBVs) or drugs. TBVs and drugs block the transmission of sexual stages found in the human blood (micro- and macro-gametocytes) to infectious sporozoites in the mosquito. Transmission intervention efficacy is measured *in vitro* by determining the antibody response to the vaccine. Various methods can be used to include ELISA, and IFA, but the transmembrane feeding assay, a functional assay, is considered the most predictive of TBV efficacy in the field. Briefly, peripheral blood containing gametocytes from humans naturally infected with malaria is obtained. To evaluate blocking efficacy of sera samples taken from humans/animals administered with a candidate vaccine. The immune sera samples are mixed with malaria infected blood samples and fed to the laboratory-rear *An. dirus* mosquitoes. The engorged mosquitoes are maintained. Approximately 7-10 days, 5-10% of the mosquitoes are

checked for the presence or absence of oocysts. The efficacy of the immune sera inhibiting parasite development is evaluated by determining the numbers of the oocysts in the mosquito midgut compare to those of the mosquitoes fed with non-immune sera. In FY12, Malariology Section conducted the experiment to test the efficacy of 9 sera collected from rabbit immunized with a Pfs230 antigen. Due to the gradually reduce of *P. falciparum* gametocyte positive case, only one sample was tested against the immune sera in the field. However, the sera showed 100% inhibition activity against *P. falciparum* infection in the mosquitoes.

CY13 Plan: Continue to assess the transmission blocking efficacy of candidate vaccines. The immune sera from a P25, P28, and P230 samples are being prepared by the NIH and will be shipped to Malariology Section, Entomology Department for the efficacy testing in FY13.

12. Title: Screening of Antimalarial Compounds in Non-Human Primates for Blood and Liver Stage Activity

Funding Source: In collaboration with Department of Veterinary Medicine

Project Summary: To support Department of Veterinary Medicine, we provide *Plasmodium cynomolgi* (monkey malaria) infected mosquitoes and live sporozoites harvested from salivary glands for the screening of antimalarial compounds. *An. dirus* mosquitoes fed on *P. cynomolgi* infected monkeys are maintained at Entomology Department. Approximately 6-9 days or 9-11 days, 5-10% of the mosquitoes infected with *P. cynomolgi* are checked for the presence or absence of oocysts respectively. Approximately 12-14 days, the sporozoites will be harvested from infected salivary glands and provided to Department of Veterinary Medicine for monkey injection. The malaria infected monkeys are used for blood schizontocidal activity and causal prophylactic activity screening of the new compounds.

CY13 Plan: Continue to provide *P. cynomolgi* sporozoites and other species of the non-human primate parasites to support several drug and vaccine development studies.

13. Title: Evaluation of *Plasmodium vivax* Susceptibility to Chloroquine in Western Thailand

Funding Source: GEIS (Sustaining and Competitive)

Project Summary: This study is the evaluation of the susceptibility of the parasite to chloroquine *in vitro*. Individuals with *P. vivax* positive blood film examinations and that met appropriate criteria were enrolled. Blood was collected from *P. vivax* infected prior to treatment for *in vitro* drug susceptibility assay. The *P. vivax*-infected blood was be incubated with different concentrations of the drug for 40 hours. Susceptibility to the drug was determined from schizont maturation inhibition efficacy. Seventy four percent (74%) of the samples went to full maturity.

We conducted *in vitro* chloroquine sensitivity tests following the method by Chotivanich *et al.* with some modification. Five fresh isolated *P. vivax* infected blood samples were collected from malaria infected patients. The samples were tested against different concentrations of chloroquine i.e., 0, 3.9, 7.8, 15.6, 31.2, 62.5, 125 ng/ml, and 250 ng/ml and then incubated at 37ºC with 5% CO_2 for 24 and 40 hrs. Thick and thin films were made and complete schizogony of parasites was evaluated per 200 infected red cells. A total of 4 out of 5 blood samples collected from patients with *P. vivax* malaria were analyzed by microscopic schizont maturation inhibition assay (40 h).

CY13 Plan: Continue to optimize *in vitro* culture system for *P. vivax*. Optimize through-put method to replace microscopic examination. Continue to evaluate susceptibility of *P. vivax* to chloroquine.

14. Title: Maintain Entomological Support for the Malaria Challenge Model

Funding Source: Collaboration with Department of Immunology and Medicine

Project Summary: We provided mosquitoes with consistent, reproducible salivary gland infections to support *Plasmodium vivax* sporozoite challenge studies either as live *P. vivax* sporozoite-infected mosquitoes and/or harvested, purified *P. vivax* sporozoites (on wet or dry-ice) to WRAIR/NMRI investigators or collaborating institutions. We also provided slides of blood and exoerythrocytic stages of *P. vivax* parasites for vaccine studies. We collected blood samples from malaria positive patients after blood smears confirmed by Ministry of Public Health (MOPH) microscopists. As part of an approved Human Use Protocol, Department of Entomology personnel are allowed to draw 20 ml of a patient's blood to feed mosquitoes using a membrane feeding technique. Studies were conducted weekly at Mae Kasa and Mae Sot malaria clinics. Aliquots of each blood sample were spotted on filter paper, and smeared on glass slides. Confirmation of parasite species was accomplished by PCR of filtered blood and by microscopic examination of blood smears. Infected mosquitoes were returned to AFRIMS and maintained in the AFRIMS insectary. Five to 10% of mosquitoes from each mosquito feed were checked for the presence or absence of oocysts approximately 7-10 days after infection. These mosquitoes were thereafter available for use in malaria sporozoite challenge studies. In October 2010, we provided *Plasmodium vivax* -infected mosquitoes for the first-ever human challenge study performed by the Department of Defense involving a candidate malaria vaccine.

CY13 Plan: Project pending. Recently, this project is being re-established in Thailand. The proposal has been re-submitted for MIDRP F funding in FY13 by Department of Immunology and Medicine.

15. Title: Active Surveillance for *P. falciparum* Drug Resistance with Assessment of Transmission Blocking Activity of Single Dose Primaquine in Cambodia

Funding Source: Collaboration with Department of Immunology and Medicine (GEIS)

Project Summary: This study is a two-arm open label treatment study comparing the efficacy, safety, tolerability and pharmacokinetics of three-day course of Dihydroartemisinin-Piperaquine (DP) with or without single-dose primaquine (PQ) in patients with uncomplicated *P. falciparum* malaria at selected sites of malaria drug-resistance in Cambodia. On the last day of DP therapy, volunteers will be randomized to receive either a single dose of PQ or DP only (no PQ). Resistance to DP and DP-PQ will be assessed by a combination of clinical, pharmacologic, and parasitological parameters including genomic signatures of selection during careful weekly follow-up visits for 42 days. Investigators will also evaluate the effect of PQ on the asexual stages of malaria parasite and potential transmissibility of infection to *Anopheles dirus* mosquitoes as compared to those not treated with PQ using membrane feeding assay.

This is an opportunity for technology transfer and capacity building in the development and implementation of the membrane feeding assay. Malariology Section provided the training to the identified personnel from the community, RCAF and/or CNM Cambodia. We also provided training in the maintenance of colony reared *An. dirus* mosquitoes, membrane feeding for mosquito infection, midgut dissection, oocyst enumeration and preservation, and sporozoite collection techniques for molecular analysis to staff of the Department of Immunology and Medicine prior to commencement of this. We provided on site assistance in the establishment of a field site laboratory for this study in Cambodia. Ten *P. falciparum* infected patients were enrolled and membrane feeding had been performed. The results are being analyzed.

CY13 Plan: Continue and complete the study in FY13.

16. Title: Evaluate Protective Immunity of the Pre-Erythrocytic Vaccine Antigen CelTOS in a Non-Human Primate Model against *P. knowlesi* Sporozoite Challenge

Funding Source: Collaboration with Department of Immunology and Medicine

Project Summary: The close phylogenetic relationship between human and non-human primates makes simian malaria infection an attractive model for studies into host-parasite interactions and vaccine induced immunogenicity. Experimental infection of non-human primates with simian malaria parasites offers a controlled system to evaluate protective immunity and malaria infection. Asiatic monkeys can be infected with several plasmodial species; i.e. *Plasmodium inui, P. fragile, P. fieldi, P. coatneyi, P. cynomolgi, P. simiovale* and *P. knowlesi.* Several documented cases of *P. knowlesi* infected humans have been reported. To better understand the mechanisms of protective immune responses induced by candidate vaccines, it is critical to use an animal model in which the immune system closely mimics that of humans. In order to test the efficacy of candidate vaccines, a challenge animal model is needed.

In this study, Malariology Section developed a protocol for *P. knowlesi* challenge by mosquito bite, and provided *P. knowlesi* infected mosquitoes used to verify the sporozoite challenge model.

An. dirus mosquitoes were fed on *P. knowlesi* infected monkey donors. The mosquitoes were infected with the parasites 100% with >10,000 sporozoites per mosquito. For the preliminary study, two naive monkeys were randomly allocated for the validation study. Five *P. knowlesi*-infected mosquitoes were allowed to feed on each monkey, and each monkey was examined for infection by measuring blood parasitemia. Both monkeys became infected. To evaluate to CelTOS vaccine efficacy, 18 monkeys immunized with the candidate vaccines were challenged with *P. knowlesi* infected mosquitoes bite. The immunized monkeys were then challenged by infected mosquitoes feeding. Data are being analyzed to determine the efficacy of the vaccine candidates, and will be published.

CY13 Plan: N/A

17. Title: Detection of *Plasmodium vivax* Using Rolling Circle Amplification (RCA) Assay in the Liver Stage Culture

Funding Source: Collaboration with DRD Section, Entomology (ILIR)

Project Summary: Malariology Section Provided *P. vivax* sporozoites for *in vitro* liver stage parasite development. Laboratory reared *An. dirus* mosquitoes that are human pathogen-free, were transported from the AFRIMS Insectary to the field site. Mosquitoes were fed on re-suspended *P. vivax* infected blood using artificial membrane feeding methods. The mosquitoes that successfully fed on *Plasmodium* infected blood samples were kept in separate containers clearly labeled with the donor identification code. Blood fed mosquitoes were returned to AFRIMS and maintained in the laboratory of the Malariology Section. Approximately 7-10 days and 14-20 days post feeding; the *P. vivax* infected mosquitoes were assessed for oocysts and sporozoites respectively. Sporozoites isolated from infected salivary glands were used for liver cell infection and the RCA assay.

CY13 Plan: N/A

18. Title: Surveillance of Pathogenic *Leptospira* Strains in Rodent and Human Populations Responsible for the Observed Trends in Human Cases of Leptospirosis in Northeast and Central Thailand and GIS/Modeling for Determination of Transmission Risk Factors

Funding Source: GEIS Competitive

Project Summary: Leptospirosis is a disease most commonly transmitted from animals to humans. Rodents are known reservoir hosts and therefore indirectly affect the epidemiology of human leptospirosis via urine contaminated with leptospires. Molecular epidemio-

logical surveillance of leptospirosis was conducted during the wet and dry seasons in Northeast and Central Thailand in 2011-2012 to identify rodent populations. The study was an attempt to determine pathogenic *Leptospira* infection rates in the rodent reservoirs and to isolate the causative leptospires from rodent kidney and/or urine. We trapped 499 rodents which were divided into 10 rodent species as follows: *Bandicota indica*, *Bandicota savilei*, *Menetes berdmori*, *Mus cervicolor*, *Mus coroli*, *Rattus argentiventer, Rattus exulans*, *Rattus losea*, *Rattus rattus* and *Tupaia glis*. The majority (50%) of the rodents caught in the Central region were *R. rattus*, whereas *R. exulans* made up 49% of the total rodents caught in the Northeast region. A total of 16% (79/499) were leptospira carriage-positive by *LipL32* real-time PCR. The rate of infection based on positive *LipL32* and partial amplification of *rrs* was significantly higher in the Central region (84%) than in Northeast region (14%). *Leptospira* infection rates found in collected rodent species were *B. indica* (20.5%), *B. savilei* (33.3%), *R. argentiventer* (30.5%), and *R. rattus* (30.5%) from the Central region, whereas the infected rodent species *B. indica* (10%), *R. exulans* (6.2%) and *R. rattus* (2.1%) were determined in the Northeast region. Directed amplification of *rrs* pathogenic *Leptospira* from rodent kidneys showed the presence of 55.7% (44/79) *Leptospira interrogans*, 43% (34/79) *L. borgpetersenii* and 1.4% (1/79) *L. kirschneri* in the r surveyed rodents. These three *Leptospira* are the most common pathogenic strain in humans. Using the Microscopic Agglutination Test (MAT), the seropositive rates of *Leptospira* in captured rodents was as high as 64%, but only 15% were considered to be carriers based on comparison of positive test results using both MAT and PCR. Phylogenetic analysis of the 16S rRNA gene revealed that 10 cultivable isolates from the Central region were closely related to *L. borgpetersenii*, while one isolate from the Northeast showed similarity with *L. wolffii*. To determine the risk of leptospira-contamination in the environment, water samples were collected from the same area in which infected rodents were found. The Central region was part of the area flooded in 2011. Of the 48 water samples, 10 were identified as uncultured bacterium clustered within a subgroup (called Clade C first found in environmental water samples in Peru) of pathogen-related/intermediate leptospira strains. From this survey, known pathogenic *Leptospira* were not detected from the environmental water samples. These results might be due to the flushing influence of the flowing water (not standing water) or the poor persistence ability of *L. borgpetersenii* within the environmental location. The high carriage rates of *L. borgpetersenii* within captured rodents suggests a strict host-to-host transmission cycle of *L. borgpetersenii* that may explain the low incidence of reported human cases but relative abundance of infected rodents in the Central region.

CY13 Plan: Continue working with collaborators with MORU (sequence typing analysis issue) and Chulalongkorn University to complete analysis of data and publish the results. Obtain additional funding to expand this study to other regions in Thailand.

19. Title: Maintenance of *Leptotrombidium* Larval Mite Colonies: Chigger-Feeding on ICR Mice (*Mus musculus*)

Funding Source: MIDRP WJ

Project Summary: Scrub typhus is an acute febrile vector-borne disease caused by *Orientia tsutsugamushi*. Larval trombiculid mites (chiggers) are important vectors of scrub typhus within Thailand and throughout the Pacific. In the chigger mite, *O. tsutsugamushi* is transmitted transovarially (from adult female to eggs) as well as transstadially (from egg to larva to adult stages of its life cycle). The department of Entomology currently maintains 7 genetically distinct strains of *O. tsutsugamushi*, each in unique populations of chiggers: *Leptotrombidium chiangraiensis* (Lc1 and Lc5), *Leptotrombidium imphalum* (L1, L2, L3 and L4) and *Leptotrombidium deliense* (Ld1). The life cycle of the *Leptotrombidium* chigger begins with the adults laying eggs. Eggs will develop to the deutovum stage. After emerging from the eggs, the deutovum develop into larval mites (chiggers) which require tissue fluids from an

animal host to develop into the nymphal stage. Finally, the nymphs become adults. Our scrub typhus infected chigger colonies were established with wild caught *O. tsutsugamushi* infected *Leptotrombidium* mites. Infected and uninfected *Leptotrombidium* feed on tissue fluids from ICR mice. Research is focused on mite biological transmission; interactions among pathogen strains (*O. tsutsugamushi*); vector competency; interactions between vectors and host; effects of multiple pathogen strains on transmission and infection rates; potential transmission blocking; and vaccine challenge trials.

CY13 Plan: Continue with current research is support of MIDRP, and GEIS goals. Expand support of collaboration project with MORU to establish a non-human primate model for scrub typhus investigations. Expand support and collaborative projects with other researchers working on scrub typhus prevention and elimination.

20. Title: Maintenance of the Scrub Typhus Chigger Challenge Model Utilizing *Orientia tsutsugamushi* Infected *Leptotrombidium* Chigger-Mite Colonies

Funding Source: MIDRP WJ

Project Summary: This is the only DoD laboratory which maintains *O. tsutsugamushi* infected mite colonies and, to our knowledge, is the only *O. tsutsugamushi* infected mite colony in the world. We currently maintain four species of *Leptotrombidium* mites with 12 infected lines: *L. chiangraiensis* (Lc) (4 lines); *L. deliense* (Ld) (1 line) and *L. imphalum* (Li) (7 line).

O. tsutsugamushi infected mite colonies are maintained in an Animal Biosafety Level-3 (ABSL-3) containment facility. The procedures are conducted in the BSL-3 facility for the infected colonies as well as in the BSL-2 laboratory for the non-infected colonies. These mites have a complex 3-4 month life cycle during which *O. tsutsugamushi* is maintained transovarially and transstadially. Only the larvae (chigger) are parasitic, feeding on extra-cellular fluid of their mammalian hosts in order to develop to the next stage. We are rearing 80,000 mites/year. Standard protocols are used to raise both *O. tsutsugamushi* infected and non-infected *Leptotrombidium* mites to support:

1. Chigger challenge model
2. Scrub typhus immunopathophysiology model in nonhuman primates (STIMP)
3. Comparative chigger sialome
4. Microbiome

CY13 Plan: Maintain the colony and establish cooperatives to further utilize this unique asset.

21. Title: Scrub Typhus Immunopathophysiology Model in Non-Human Primates (STIMP)

Funding Source: National Institute of Health-IAA

Project Summary: Overall objective is to establish a non-human primate model to more closely mimic the immunopathophysiological parameters of natural infection with, and vaccine-induced protection against scrub typhus. This project is a collaboration of researchers from AFRIMS, Navy Medical Research Center (NMRC) and Mahidol Oxford Research Unit (MORU). Department of Entomology, AFRIMS is responsible for establishing effective mechanical injection of mites using the human pathogenic Karp strain of *O. tsutsugamushi*. The objective is to determine the most suitable position on the primate to achieve transmission using optimum mite infection rates established in the mouse model. The advantage of this study is the nonhuman primate pathophysi-ology more closely mimics humans, and will produce more accurate conclusions and decision points for vaccine challenges.

CY13 Plan: Support transition of current mouse chigger challenge model to the non-human primate model.

22. Title: Evaluation of r56 Vaccine Candidates in Homologous Chigger Challenge Mouse Model

Funding Source: MIDRP WJ

Project Summary: The specific aim of this project is to evaluate the protective efficacy of recombinant protein r56 in homologous a chigger challenge mouse model. This is a follow-on study to an earlier MIDRP project, "Establishment of a Chigger Challenge Model. Determination of Optimum Inoculation Doses of Two *Orientia tsutsugamushi* Strains in ICR Mice", and "Evaluation of Scrub Typhus Vaccine Candidates by Natural and Artificial Challenge Models in ICR Mice (*Mus musculus*)". The overarching goal of the program is to develop vaccines to elicit broadly cross-reactive protection against infection. Therefore, knowledge of the protective ability of the vaccine candidate in a chigger challenge model is critical. One of the contributing factors for the poor protection of r56Kp immunized mice against Lc-1 chigger challenge could be homologous versus heterologous immunogens, as poor heterologous protection has been observed in nature and in the needle challenge. In order to eliminate contributing factors due to homologous versus heterologous immunogens to elucidate protective immune responses and simplify data analysis, we amplified the *Orientia* 56kDa gene from the Lc-1 infected chigger and produced the recombinant protein r56Lc-1. The protective efficacy of this r56Lc-1 will be evaluated by challenge with chiggers which are infected with *Orientia* strain Lc-1.

CY13 Plan: Evaluate r56LC-1 vaccine and natural chigger challenge model

23. Title: Mosquito Rearing for Experiment Support

Funding Source: MIDRP

Project Summary: Rear and maintain vigorous and human pathogen free colonies of seven *Anopheles* mosquito species: *An. dirus*, *An. cracens*, *An. minimus*, *An. sawadwongporni*, *An. campestris*, *An. kleini* and *An. sinensis*; 2 spp. of *Aedes* mosquito, *Ae. aegypti*, *Ae. albopictus*; and *Toxorhyncites splendens*. These mosquitoes support basic mosquito biology, pesticide resistance, and pathogen transmission efficacy studies within the Entomology Department as well as other Departments within AFRIMS, WRAIR and other collaborators. Research focus is disease elimination and prevention in Thailand, South East Asia and worldwide.

Laboratory-reared mosquitoes are provided on request are disease-free, of the same age, and in impeccable condition. The first step of rearing starts when eggs are immersed in water in larval rearing trays. After 1-2 days, eggs will hatch. On day 2 after hatching, larvae will be fed in the rearing tray with ground fish food. When pupation starts, pupae will be collected and kept in a tub of fresh water until emergence. Adults will be fed with vitamin syrup until 5-7 days old when females are needed for experiments or for natural/artificial mating to maintain the colony. Females will be fed a blood meal via an artificial membrane feeding apparatus to provide protein for egg production.

Numbers of mosquitoes produced to support 2012 experiments are recorded in the table below.

Mosquito sp.	**Number of females produced to support 2012 experiments**
An. dirus	186,110
An. sawadwongporni	4,000
An. campestris	6,800
An. minimus	25,530
An. cracens	8,100
An. kleini	8,300
An. sinensis	8,200
Ae. aegypti	7,982,000
Toxorhynchites splendens	8,580 (adultM&F)