BIONOMICS AND ECOLOGY OF *ANOPHELES LITORALIS* ON BONGAO ISLAND, TAWI-TAWI PROVINCE, PHILIPPINES: IMPLICATIONS FOR VECTOR CONTROL

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Abstract. Entomological surveys were conducted to identify *Anopheles* malaria vector species, their feeding and resting behaviors, and characterization of larval habitats on Bongao Island, Tawi-tawi Province, in July and November, 2007. Survey parameters included all-evening human-landing collections (HLC), evening buffalo-baited trap (BBT) collections, daytime indoor and outdoor adult resting collections, adult female age-grading, identification of natural *Plasmodium* infections in mosquitoes, larval habitat identification and physical/biological characterization, and adult insecticide susceptibility assays. Both surveys revealed the predominant and putative malaria vector species on Bongao Island is *Anopheles litoralis*. *Anopheles flavirostris* was collected on only one occasion. The HLC during the July survey produced approximately 4 mosquitoes/human/night (mhn). The November survey yielded 1.27 mhn due, in part, to inclement weather conditions during time of sampling. *Anopheles litoralis* host seeking behavior occurred throughout the evening (06:00 PM-06:00 AM) with peak biting between 10:00 PM and 04:00 AM. This species exhibited stronger zoophilic behavior based on comparison of HLC and BBT data. HLC showed a slightly greater exophagic (outdoor) behavior (1.4:1 ratio). During the July collection, an older adult population was present (75% parous) compared to the lower numbers of *An. litoralis* dissected in November (25% parous). Albeit a small sample size (n=19), 10.5% of *An. litoralis* dissected contained midgut oocysts of *Plasmodium*. Daytime adult resting harborages included biotic and abiotic sites in and around partially shaded, brackish water habitats where immature stages were common. *Anopheles litoralis* was found susceptible to pirimiphos-methyl and four different synthetic pyrethroids. This survey provides further epidemiological evidence of the importance of *An. litoralis* in malaria transmission on Bongao Island, and presumably throughout much of the Sulu Archipelago in the southern Philippines. Published observations of this species remain very limited and further investigations on the bionomics

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and epidemiological importance of this species are needed. Both ecological and human factors in malaria transmission are presented with implications for improved control of *An. litoralis* and prevention of infection.

Keywords: *Anopheles litoralis*, bionomics, Tawi-tawi, Sulu Archipelago, Philippines

INTRODUCTION

Malaria control interventions strive for a reduction in human morbidity and mortality using the most efficacious and cost-effective methods possible. The Philippine National Malaria Control Program aligns its vector control strategies on the site-specific bionomics of the major vector species present, most notably *Anopheles* (*Cellia*) *flavirostris* (Ludlow). Studies conducted by the Research Institute for Tropical Medicine (RITM), Alabang (near Manila), have shown behavioral variations among *An. flavirostris* populations in the provinces of Bataan and Quezon in Luzon and Agusan del Sur in Mindanao Island. Differences in time of peak biting densities, blood feeding activity and resting behavior have been described for this species (Salazar *et al*, 1988; Salazar, 1989; Asinas, 1993). Insecticide-treated nets (ITN) and indoor residual sprays (IRS) remain the primary vector control interventions in many parts of the country endemic for malaria. Similar vector control strategies have been implemented on the main islands of the Sulu Archipelago (Basilan, Sulu, and Tawi-tawi) in the southernmost part of the country. These relatively small islands present somewhat unique ecologies from the rest of malarious regions of the country as manifested by the common presence and medical importance of the predominately brackish water vector species, *Anopheles (Cellia) litoralis* King (King, 1932; King and Del Rosario, 1935; Cabrera *et al*, 1970; Darsie and Ramos, 1970; Hii, 1978; Catangui *et al*, 1985; Miyagi *et al*, 1985; Tsukamoto *et al*, 1985).

Anopheles litoralis is a member of the Pyretophorus Series, which also includes some of the most efficient malaria vector species in the world (*eg*, Gambiae Complex in sub-Saharan Africa and the Sundaicus Complex in Southeast Asia). This species is biologically similar to *Anopheles sundaicus* sensu lato, a species complex present throughout much of Southeast Asia, of which it closely resembles morphologically except for the presence of a prehumeral pale spot on the wing of the former (Reid, 1968; Cagampang-Ramos and Darsie, 1970). *Anopheles litoralis* is found commonly and abundant along the coasts of the entire Philippines but has only been implicated as an important malaria vector on Palawan Island and south of Luzon in the provinces of Mindoro, Samar, Surigao, Zamboanga, Basilan, and the Sulu Archipelago (Catangui *et al*, 1985; Salazar, 1989, EPMU6, 1990). This species has been identified as the sole malaria vector in Pangutaran and Tatalan islands in Sulu and shown capable of transmitting both *Plasmodium falciparum* and *Plasmodium vivax* parasites (Cabrera *et al*, 1970; Catangui *et al*, 1972).

Anopheles litoralis appears restricted to the coastal areas of the Philippine Archipelago and Sabah (northern Borneo), Malaysia (Bonne-Wepster and Swellengrebel, 1953; Hii, 1978). Adult mosquitoes have been recorded as far inland as 32 km from known larval habitats (Baisas, 1972). Typically associated with brackish water marshes and lagoons, rock pools and crevices in exposed coral reefs, this species can also frequent human-made brackish water fishponds and salt evaporation beds (Salazar, 1989). It is well adapted to surviving in sea water and high salinity conditions (≥2.5% to 3%) in the drier months of the year. Common habitats are often associated with abundant floating algal growth. The species can often be found year-round so long the proper environmental conditions and salinity levels are maintained (King and Del Rosario, 1935). Adults have been recorded resting indoors and outdoors including interior earthen wells and at the base of vegetation (Basio, 1971). Human biting activity has shown females to be most active during the first quarter of the evening (08:00-09:00 PM). A species known to feed on both humans and other animals, it is regarded a primarily zoophilic species throughout its range while having a greater preference to blood feed outdoors on both human and animals depending on the availability of either (Baisas, 1972; Catangui, 1985).

Malaria remains an important disease in many areas of the Philippines, especially in Palawan Island and many more remote southern islands of the country. The disease is endemic on the larger island groups of the Sulu Archipelago. Malaria has long been recognized as a major impediment to economic development in Tawi-tawi Province. During 2004-2007, Tawi-tawi contributed an annual average of 6,408 reported malaria cases, making the province as a whole among the top five malaria endemic provinces in the country. These scattered and relatively remote islands possess unique vector ecologies from the remainder of the country. There remains a relative paucity of information on the bionomics and ecology of the primary malaria vectors, particularly *An. litoralis*, in these southern-most regions, including susceptibility to residual insecticides currently used in the national malaria control program.

The limited descriptions of the ecology and bionomics of *An. litoralis* in Tawi-tawi Province has been a major impediment to a better understanding of the malaria transmission dynamics. The susceptibility status of adult mosquitoes to pyrethroid insecticides applied on the interior walls of houses and treated bed netting has been long neglected. The findings of such investigations would assist in the development of more site-specific and appropriate approaches to vector control as outlined in the national policies for the control of malaria vectors (Philippine National Malaria Control Program, 2009).

The primary objective of this study was to characterize the bionomics of the potential and putative malaria vectors on Bongao Island and assess the implications of these findings on currently implemented and recommended vector control on the island. The specific objectives included: 1) identification of anopheline species present, their relative densities and geographical distribution at the time of collections, 2) determine malaria vector status of each species via dissection of midgut and salivary glands, 3) describe the larval habitats and adult indoor-outdoor resting areas, 4) document adult blood feeding behavior based on host availability and environment, and 5) determine age structure of vector population at time of collections.

MATERIALS AND METHODS

Study sites

The entomological surveys were conducted in July (normal wet season) and November 2007 (the typical dry time of the year) in barangays (villages) Tubig Basag and Swangkagang, in the municipality of Bongao Island, the administrative

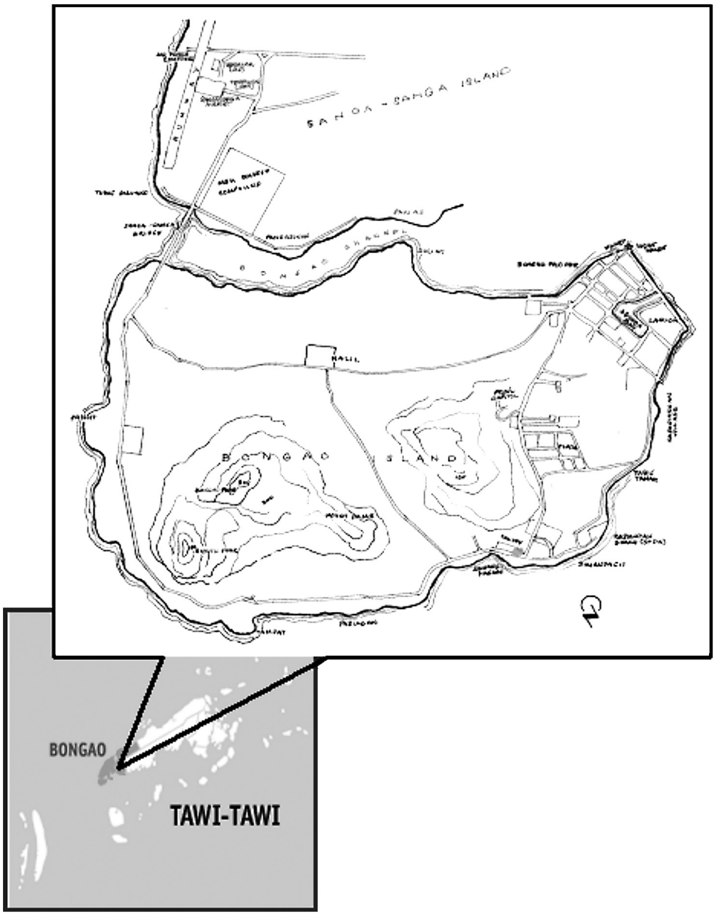

Fig 1–Municipality of Bongao Island, Tawi-tawi Province, Republic of the Philippines.

capital and commercial center of Tawi-tawi Province located at the southwestern tip of the Tawi-tawi Island group (5°5′15″ S; 119°44′120″ E) (Fig 1). Bongao municipality is surrounded by the Celebes Sea on the east and the Sulu Sea to the south. The administrative area covers approximately 25,000 ha and comprised 35 barangays located on the three largest islands, Bongao, Pababag and Sanga-Sanga. The population density base on the 2010 census (National Statistics Office Philippines) was approximately 550 persons per km^2. The terrain is generally flat with the highest elevation 300m above sea level. The economic livelihood is primarily agrarian, with agriculture (copra, root crops, fruits and vegetables), fishing and agar-agar (seaweed) cultivation the primary sources of income, while many also engaged in various barter trades.

Villages

The majority of houses in Tubig Basag (TB) and Swangkagang (SK) are located adjacent to the shore line at between 10-15 m and 45-50 m distance inland, respectively (Fig 2a, b). Houses generally sit on elevated stilts, either on permanent dry land or over shallow tidal water. The walls and floors are usually constructed of wood (commercial hardwood, bamboo or mangrove), and the roofing is either made of natural dried vegetation (Cogon grass; *Cynodon dactylon*) or galvanized iron sheets (Fig 3 a, b). Most houses are composed of a single large room, which serves as both communal eating and sleeping areas, and an attached, smaller kitchen area. Floors are typically made of wooden slats with varying degrees of open gaps between adjoining sections. The space beneath the house is often occupied by domesticated animals (*eg*, chickens, dogs, goats, etc) serving as sleeping or roosting quarters. These animals potentially provide an ample blood source for mosquitoes. Household electricity is provided by generators on floating barges and it is usually available during fair weather conditions.

Most commercial business estab-

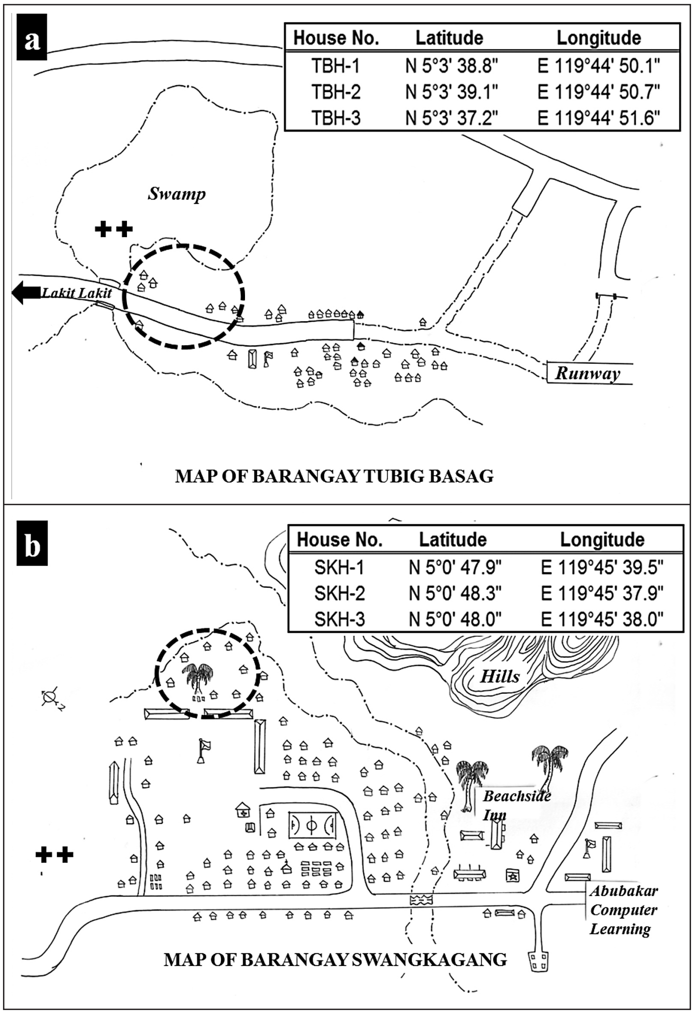

Fig 2–Map of Barangay Tubig Basag (a) and Barangay Swangkagang (b). Mosquito collection sites (3 houses each) are encircled and locations with *Anopheles litoralis* larvae are marked by '++'.

Fig 3–Typical housing construction in Bongao, Tawi-tawi.

lishments close in the early evening at around 07:00 PM. At this time the majority of adult women and children are already inside their houses preparing meals or settling in for the night. However, many adult men will continue to stay outside to participate in nighttime social activities such as drinking alcohol and gambling. This outdoor exposure likely increases the risk of contracting malaria in the adult male population.

Implementation and budgeting of the malaria control program is the responsibility of the regional and provincial health staff and local government. Existing malaria vector control interventions include periodic residual spraying of interior house walls and distribution of insecticide-treated bed nets (ITNs). In some instances, ITNs are utilized as screens or curtains across open windows.

Meteorological data and malaria cases

Bongao is located in the equatorial zone and has no pronounced dry or wet seasons and is fortunate to generally not to be impacted by significant or prolonged storm systems. In 2007, the average monthly rainfall was 179.8 mm (range 135 to 211 mm) and was fairly evenly distributed throughout the year (Fig 4). Warmest months were from March to September and

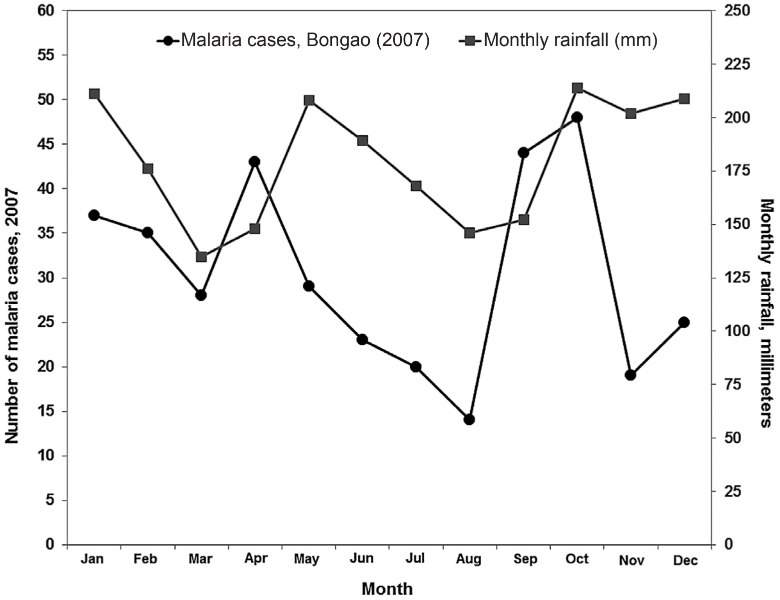

Fig 4–Monthly malaria cases in the municipality of Bongao, Tawi-tawi with reference to mean monthly rainfall in 2007.

Fig 5–Buffalo-baited trap during daylight hours.

coolest from October to February with average maximum and minimum ambient air temperatures of 30.8ºC and 23ºC, respectively (Fig 4).

Human landing collections

Human-landing collections (HLC) were performed four consecutive evenings in July and three consecutive evenings in November 2007. Collections were conducted in three houses each in TB and SK (Fig 2a, b inset) and the same houses were used both collection periods. House selection criteria were based on recently reported malaria case(s) from occupants before the survey was conducted, a willingness and consent of the households to participate, and considerations on the security and safety of nighttime collectors. Outdoor and indoor collections were performed simultaneously using 2 trained collectors beginning at 06:00 PM and ending at 06:00 AM the following morning. One collector was positioned inside the family-occupied house and another was stationed approximately 1-2 m near the outside the same dwelling. Collectors, while in a sitting position, expose both lower legs (knee to ankle) for mosquitoes to alight on. Each collector used a flashlight and mouth aspirator tube to collect all mosquito species that landed on exposed legs. The paired collectors alternated positions from indoors to outdoors at the start of the 12:00 PM collection interval to minimize potential bias in individual collector attractiveness and collection efficiency. A team supervisor ensured that the collectors remained awake the entire night. Collections occurred for 45 minutes each hour with a 15 minutes break between collection intervals.

Collected mosquitoes were combined by 2-hour intervals, placed in Styrofoam® cups fitted with cloth netting over the open end and served as temporary holding receptacles during the evening. In the morning, mosquitoes were taken to a field laboratory for morphological species identification using a dissecting microscope and published taxonomic keys (Cagampang-Ramos and Darsie, 1970). Summary calculations per night activities were based on a total of 6 person-nights collection per barangay and divided by collection location (indoor or outdoor). Evening mosquito density per collector or mosquitos per human/night (mhn) was calculated for each species based on a total of 10.5 hours of actual collection time per person and adjusted to 12 hours to represent an estimated attack density for a full night.

Buffalo-baited trap collections

Buffalo-baited trap (BBT) collections (Fig 5) were carried out simultaneously with the HLC each night in the same villages. The BBT locations were chosen on the same basis as the HLC houses, *ie*, relative proximity to potential vector larval habitats and recent malaria cases. BBT collections were performed at approximately 500 m distance from the nearest HLC station using a single adult water buffalo (*Bubalus bubalis carabanesis*) per net trap per village. BBT nets were set in areas near known vector breeding sites at between 1 and 5 m from the edge of brackish water mangrove swamps, often beneath an adjacent mature coconut grove. The buffalo was kept tethered overnight inside the mosquito net with one section of the net remaining open to let mosquitoes freely enter. The net was closed just before scheduled hand collections each hour beginning at 06:45 PM. All mosquitoes were collected manually from the inside surface of the net using a flashlight and mouth aspirator. Modified cups, as described for the HLC activities, served as temporary holding receptacles for mosquitoes during the evening. Mosquito collections were kept separated by each hour of collection and location and transferred to the field laboratory for species identification at the end of the collection period. In July, 4 BBTs were in use compared to only 2 in November.

Adult resting collections

Collections of resting, freshly-blooded adult anophelines were conducted indoors and from nearby outdoor sites of same house at 15-minute intervals each hour during intervals between HLC activities. Outdoor searches did not exceed a 3 m distance from the house. Inspections included outside house walls, drift wood, pots, makeshift planters, plants and trees near the house within collector's reach. Indoor collections were made in various rooms of the house designated by the homeowner. All collections were made using mouth aspirators with the aid of a flashlight. Adult resting collections took placed for a total 1.5 hours each evening by a separate set of trained collectors. All mosquitoes were segregated and processed using the same procedures as HLC collections. The collected mosquitoes were grouped by 2-hour intervals and were brought to the laboratory the following morning for identification and processing.

Daytime adult resting collections were conducted around vegetation and other possible harborages located within 500 meters from known active larval habitats. Resting adults were collected using mouth aspiration tubes and placed in holding cups until identification. Sweep netting was also performed to augment visual inspection.

Age-grading

Human-bait collected *Anopheles litoralis* were dissected each collection to determine parity status following a standard methodology (WHO, 1994 a,b). Approximately 20% of females from each 2-hour interval were selected randomly for dissection. The female was anesthetized using ether vapor or placed in a freezer at minus 20°C for 3-5 minutes until immobilized. Using a dissecting stereomicroscope, ovaries were removed and placed on a glass microscope slide and allowed to air dry. Viewing the ovarian tracheolar skeins, a determination was made of parity status as either nulliparous or parous (Detinova, 1962). Females with advanced ovarian development (eggs) were recorded as parous. Parity determinations were limited to females with stage I or II ovarian development.

Plasmodia infections in mosquitoes

From a random selection of *An. litoralis* captured by HLC, the midguts and salivary glands were extracted and placed onto separate slides to determine presence of oocysts and sporozoites (WHO, 1975). Dried samples were fixed with alcohol, rinsed with water and stained with Giemsa stain (1:20 dilution) for 40 minutes. Specimens were washed and dried prior to examination using a light compound microscope.

Larval habitats

Surveys for immature stages were carried out to identify and characterize suitable and active *Anopheles* aquatic habitats in and around the two study sites. Water bodies were sampled for immature stages using plastic dippers. Coordinates of sample locations were recorded using a hand-held global positioning system device (Garmin GPSMAP 76, Olathe, KS). A topographical, ecological and physical description of the larval habitats, the surrounding area, type of vegetation, and presence of potential aquatic predators were recorded.

For physical parameters, water samples were taken for analysis of dissolved oxygen, pH, nitrites, salinity and turbidity. The dissolved oxygen of the water near the BBT area was determined through a titration method (USEPA, 2007). Water was collected near algal mats found harboring *An. litoralis* larva between 06:30-07:00 AM. For better accuracy, testing for dissolved oxygen was conducted within 30 minutes of collection. Nitrite (nitrogen), pH, and salinity levels were determined by an independent laboratory (Institute of Chemistry-Analytical Chemistry Section, University of the Philippines, Los Banõs). All sample collections were done in triplicates and results are expressed as means.

Insecticide susceptibility assays

For susceptibility bioassays, 5 different insecticides (1 organophosphate and 4 pyrethroids) were compared using insecticide impregnated papers produced by the Vector Control Research Unit, Universiti Sains Malaysia, Penang, Malaysia based on WHO specifications and designated 'diagnostic' operational concentrations. Insecticide-impregnated papers and non-insecticide control papers were placed inside respective exposure tubes.

Fifteen wild-caught, blooded female *An. litoralis* mosquitoes derived from BBT collections were used per test. A total of 75 mosquitoes were examined for each insecticide concentration with matching non-insecticide controls in a series of 5 assays each. Mosquitoes were placed in clean holding tubes for 1 hour before insecticide exposure to detect any damage due to handling; those mosquitoes found moribund or ailing were excluded from the final analysis. The specimens were then transferred to the tubes containing insecticide-treated papers and exposed for 1 hour. Knocked-down mosquitoes were recorded at 5-minute intervals during the duration of the test. After the exposure period, mosquitoes were transferred to clean holding tubes and provided with 10% sugar solution on cotton balls and observed following a 24-hour holding period to record the final mortality for insecticide-exposed and control mosquitoes.

Data analysis

Data was collated for analysis at the Department of Medical Entomology, Research Institute for Tropical Medicine. The mosquito density per human was calculated by summing the numbers collected over each night and each site of collection to derive an average number of mosquitoes per person per night for each location (house, indoors or outdoors). The susceptibility status of mosquitoes to each insecticide was evaluated based on revised World Health Organization criteria (WHO, 2013). If necessary, final test mortality was adjusted against matched control mortality using Abbott's formula (WHO, 1981). For insecticide susceptibility assays, probit analysis (Finney, 1971) was used to analyze knockdown (KD) time during the 1-hour exposure. KD_{99} (knockdown of 99% of exposed sample) was computed from the susceptibility tests to serve as reference ('baseline') for comparison with future resistance evaluations in the same populations.

RESULTS

Mosquito identification

Anopheles litoralis was one of 11 anopheline species captured in Bongao and the most common species found in both villages during both survey periods. In comparison to the major malaria vector throughout most of the Philippines, *Anopheles flavirostris*, is larger in size and very easy to morphologically distinguish from the latter.

Human-landing collections

HLCs collected a total of 1,478 mosquitoes during the 2 survey periods. *Culex* mosquitoes comprised 1,178 or 79.7% of the combined collection, with *Cx. quinquefasciatus* as the dominant species. Among anophelines, *An. litoralis* was the dominant species with 188 individuals or 12.7% of entire collection and 98% of all anophelines recorded. Only 3 other *Anopheles* species were collected on human bait, a single female specimen of *An. flavirostris* during the November survey in SK, *Anopheles tessellatus* Theobald (2=0.14%) and *Anopheles samarensis* Rozeboom (1=0.07%). Dengue and Chikungunya virus vectors were also recorded using HLC; *Aedes aegypti* (L.) (=*Stegomyia aegypti*) (56=3.79%) and *Aedes albopictus* Skuse (=*Stegomyia albopicta*) (53=3.59%) or 7.4% of total. HLC revealed the presence of *Ochlerotatus poicilius* (Theobald), a suspected vector of nocturnally periodic Bancroftian filariasis in the Philippines (Biasas, 1972).

In both villages, *An. litoralis* began biting at dusk and continued throughout the night until end of collection at 06:00 AM. In July, the combined peak attack rate was between 12:00 PM and 04:00 AM; however,

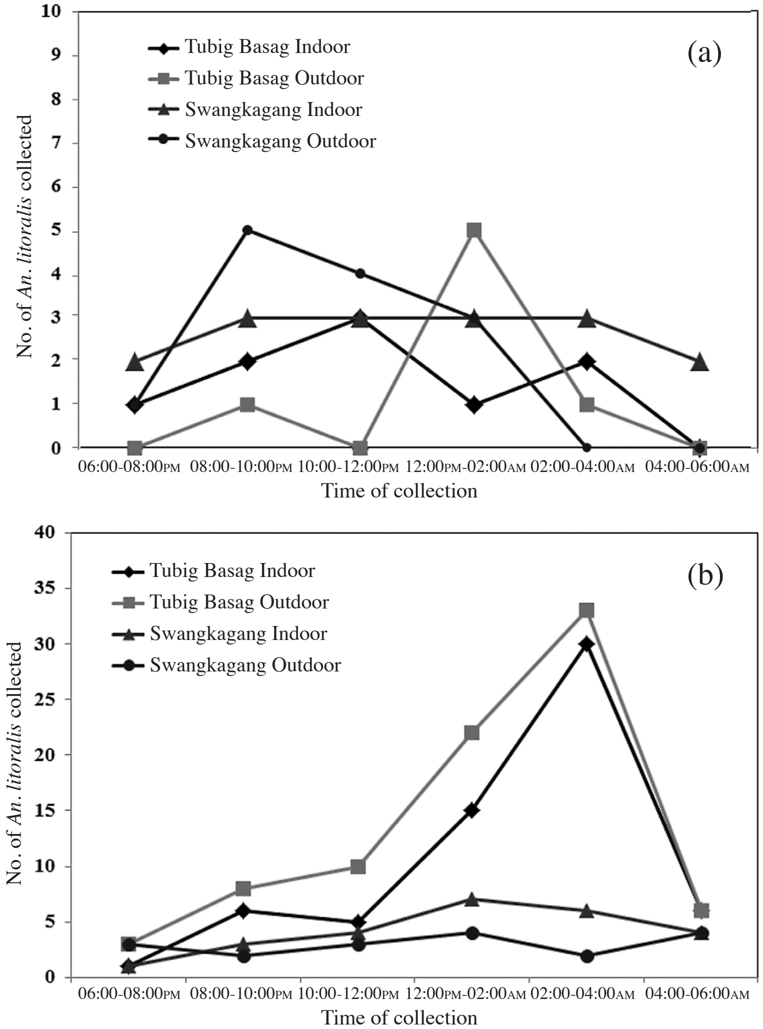

Fig 6–Comparison of indoor and outdoor *Anopheles litoralis* HLC from Tubig Basag and Swangkagang, July 2007 (a) and November 2007 (b).

peak landing times differed between the two village clusters. Peak densities in TB were more pronounced from 02:00 and 04:00 AM, whereas SK saw peak activities slightly earlier between 12:00 PM and 02:00 AM (Fig 6a). In both villages, there appeared a slightly greater exophilic tendency with a 1.4 to 1 outdoor to indoor ratio from all collections combined. The second survey in November found lower numbers of *An. litoralis* compared to July. Moreover, the peak biting times were not as well-defined compared to July, likely due to the smaller sample size, and showing greater variations in the indoor and outdoor data per site (Fig 6b).

An overall human landing density for *An. litoralis* was 5.2 mhn (n=188). The July survey revealed higher densities (8.05 mhn) of *An. litoralis* in TB than SK (2.38 mhn). The second survey had a combined lower density of 1.7 mhn. Overall densities were 0.96 mhn and 2.44 mhn for TB and SK sites, respectively. This can be attributed, at least in part, to the unexpected poor weather conditions experienced during the collection period in November. There were heavy rains and strong winds due to presence of an unseasonal typhoon system in the area.

Buffalo-baited trap collections

The July and November surveys showed similar species distribution and number of mosquitoes per animal. In July, 564 mosquitoes were collected (mean 141 per animal) consisting of 307 *Culex* spp (54.4%), 246 *Anopheles* (43.6%) and 11 *Aedes* (1 *Ae. albopictus*; 1 *Ae. aegypti*, 9 *Ae. poicilius*). Similar to the HLC findings, the most common anopheline captured was *An. litoralis* (n=142) representing 25.2% of all mosquitoes and 57.7% of all anophelines captured. The November collection produced 276 mosquitoes (mean 138 per

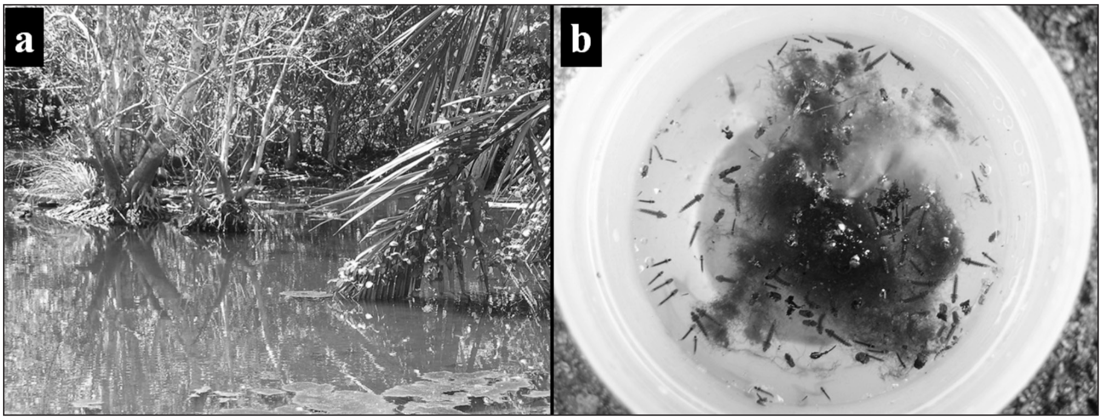

Fig 7–Larval habitats of *Anopheles litoralis*: brackish water swamp (a), floating algae with immature stages collected from same location (b).

animal) of which *Anopheles* comprised 82.2% (227) of the entire collection while only 48 (17.8%) were culicines (1 *Ae. albopictus*). *Anopheles litoralis* was the dominant species (n=182), at 65.9% of overall collection and 80.2% of all anophelines. The nine other anopheline species included: *Anopheles indefinitus* (Ludlow), *Anopheles kochi* Doenitz, *Anopheles limosus* King, *Anopheles ludlowae* (Theobald), *Anopheles philippinensis* Ludlow, *An. subpictus* Grassi, *An. vagus* Doenitz, *An. samarensis*, and *An. tessellatus*.

Host preferences

All collection data from BBTs were combined from both sites and compared with combined HLCs. Together, *An. litoralis* showed a greater zoophilic tendency. BBTs produced a mean of 45.5 (n=182/4) and 71(n=142/2) mosquitoes per animal/night in July and November, respectively, while the matching landing densities were 5.20 mhn and 1.7 mhn during the same periods. BBT rates were 8.75 and 41.7 times higher than HLC for the first and second surveys, respectively. Fewer complete trap nights were completed in November (3 full nights) due to unfavorable weather conditions.

Age-grading

Anopheles litoralis species were randomly selected from each evening HLC in July and November. July showed a total parous rate of 75% with higher parity observed from TB (92%; n=26 dissections) compared to SK (53%; n=19). A lower number of *An. litoralis* were collected during November and were found to be a relatively younger population (25% parous), with TB and SK samples 100% and 70.6% nulliparous, respectively.

Oocyst and sporozoite infections

From 19 *An. litoralis* dissections, 10.5% were found infected with malaria oocysts on the midgut. Salivary gland dissections did not reveal evidence of sporozoites. Absence of non-human primate species in Bongao Island ruled out the possibility of parasites coming from a non-human origin. Additional testing (*eg*, circumsporozoite ELISA or PCR) was not done to either confirm or determine *Plasmodium* species.

Adult resting behavior

A total of 76 blood-fed *An. litoralis* were captured in evening resting collections. Combined data from the two survey periods showed a greater exophilic (1.4:1 outdoor to indoor ratio) in both villages. Indoor resting places included walls, and under benches and tables. Outdoor collections were more productive from underside of house eaves, outside walls and surrounding vegetation proximal to house collection stations.

For daytime adult resting collections, the most common potential harborages inspected included outside hanging plants near houses, logs, tree holes, Nipa palm (fruit and fronds), coconut husks, and other debris in the immediate vicinity of HLC and BBT collection sites. The dominant vegetation in the vicinity of known vector breeding sites were Nipa palm, *pandan* grass, coconut trees, aquatic plants like *kaong* (sugar palm fruit) trees, mangroves, ground ferns, lantana plants and various other tree and brush saplings.

Collections revealed the following as *An. litoralis* day-resting harborages: dried ferns along the shore edge of a brackish water 'swamp' near a BBT site, dried wood on the ground near the edge of the swamp, inside cracks of coconut husk hanging about 1 m from the ground used as containers for orchids, tree holes, branch holes in drift wood used as plant holders, dried plant branches and banana plants near a BBT site, coconut shells from copra harvesting, ornamental plants and a papaya tree near a HLC house. Other insects were collected from sweep netting and BBT, included dragonflies (Anisoptera) and damselflies (Zygoptera), walking stick (Phasmida), a variety of adult hemipterans, coccinellids (and nymphs), *Pachyrhynchus* weevil, chrysomelid (leaf beetles), cetonilid (flower beetles) and twig-cutter beetles, various dipterans including *Aedes albopictus*, deer flies (Tabanidae) and *Sarcophaga* (flesh flies) sp, hymenopterans including vespertilionid wasps, *Thyreus* sp (Cuckoo bee), various lepidopterans, millipedes and spiders.

Larval habitat ecology

Bongao Island lacks perennial sources of freshwater from springs, small streams or rivers. Rainwater is apparently the only source of freshwater in TB with groundwater wells claimed as salty and generally unpalatable. Regular mixing of fresh and salt water was observed when sea waves entered nearby swamp areas during high tides. A large swamp containing Nipa palms and mangrove was located about 40 m from a cluster of houses in TB used as mosquito collection stations. The swamp had a soft mud floor; visibility estimated at 10-12 cm from the water surface. There were relatively large numbers of *Anopheles litoralis* larvae found in these brackish habitats. The areas with more concentrated *An. litoralis* larvae were partially shaded and often contained floating natural debris and vegetation. Patches of floating algal mats (*Enteromorpha* sp) were present and commonly associated with *Anopheles* larvae and pupae. Most larval habitats were exposed to direct sunlight for varying periods of the day. Many households were located near the swamp areas containing *An. litoralis*. Unlike TB, SK had an absence of large, perennial swamps nearby. The closest potential breeding site was a nearly dried, man-made channel that contained a mixture of fresh rainwater and salt water from high tides. The cluster of houses at this site was more compact than the site in TB. The major source of freshwater for the village is also rainwater. This difference in household density between the two village sites relative to larval positive locations is shown in Fig 2a, b.

Potential predators of mosquito larvae in habitats sampled were various small sized fish species (goby mudfish and marine fingerlings), water striders (Gerridae), water boatmen (Corixidae), and backswimmers (Notonectidae). Dragonfly and damselfly naiads were not observed in collections although adult insects were seen active in the area.

Physico-chemical characteristics of coastal swamp breeding sites in TB were measured. Of sampled breeding sites, results found *An. litoralis* is capable of surviving within a range of 2.7 to 3.2 mg/l oxygen (2.7-3.2 ppm), a mean of 2.97 mg/l, or the near equivalent of 40% saturation of dissolved oxygen in the sampled water. Surface water samples were either made just beneath or very near the edges of algal growth patches in conditions varying from no, slight to intermittent rainfall. Oxygen analysis showed that beneath the algal growth, without rain, 3.0 mg oxygen/l; beneath the algal growth with intermittent rain, 3.2 mg/l, and near the algal growth with slight rain, 2.7 mg/l. Hydrogen-ion activity (pH) was near neutral (pH 6.97), salinity (low at 0.04%), alkalinity (187.7 mg/l $CaCO_3$, and rated as 'hard to very hard' on the water hardness scale), and dissolved nitrate (low nitrogen content at 0.003 mg/l). Although *An. litoralis* is known to tolerate, possibly even prefer, much higher percent salinity, the aquatic sites were found to be only slightly saline at time of sampling. A low salinity level of 0.04% appears acceptable for *An. litoralis* survival but more data is needed to confirm the optimal salinity and maximum tolerance for immature development to successful adult emergence.

Lastly, the reported malaria cases were found to be randomly distributed throughout each month and did not show obvious or statistical associations with any of the measurable climatic variables (rainfall, relative humidity, minimum and maximum air temperatures).

Adult insecticide susceptibility assays

Susceptibility assays of *An. litoralis* against pirimiphos-methyl (organophosphate), and deltamethrin, permethrin, etofenprox and lambda-cyhalothrin (pyrethroids) was conducted during the November survey. A total of 75 female *Anopheles litoralis* were exposed to each operational insecticide concentration. Table 1 shows cumulative mortality after 24 hours post-exposure to each chemical. Pirimiphos-methyl showed low knockdown with only 1 to 2 individuals found moribund at the end of 1-hour exposure; whereas the 24-hour post-exposure mortality was 100%. Consistent knockdown responses were seen in all replicates for etofenprox beginning after 25 minutes exposure. The other three pyrethroids showed more rapid knockdown between 5 and 15 minutes into the test. Zero knockdown and mortality was observed in the controls. *Anopheles litoralis* was found completely susceptible to pirimiphos-methyl and deltamethrin, while showing suggestions of the existence of low-grade resistance to permethrin, lambda-cyhalothrin and etofenprox. The susceptibility status to each insecticide was based on recently revised WHO criteria (WHO, 2013), *ie*, mortality between 98% and 100%, a test population is considered "susceptible"; mortality between 90% and 97% is treated as "suggestive of the existence of resistance" and requiring further investigation such as additional bioassays or determination of mechanism(s); and mortality below 90% is an indication of outright "resistance" in the population. The WHO further recommends that if the observed mortality is between 90% and 97%, then either the presence of resistant genes in the vector population should be determined or at least two additional contact tests that show mortality is consistently

Table 1
Probit analysis of knockdown responses and susceptibility of *Anopheles litoralis* to insecticides.

Insecticide	KDT_{50} min	KDT_{99} min	Percent mortality at 24-hr (%)	Interpretation
Pirimiphos-methyl, 1%	672.75	253.73	100.0	Susceptible
Deltamethrin, 0.05%	13.21	51.02	100.0	Susceptible
Permethrin, 0.75%	16.77	56.78	97.3	Possible resistance
A-cyhalothrin, 0.05%	18.26	66.03	96.0	Possible resistance
Etofenprox, 0.5%	40.79	180.17	93.3	Possible resistance

below 98%, confirms resistance is present. Based on these recommendations, our results are provisional and further tests are warranted using a larger number of samples. Table 1 shows the decreasing order of knockdown potential per active ingredient using probit analysis as: deltamethrin (0.05%) <permethrin (0.75%) <lambda-cyhalothrin (0.05%) <etofenprox (0.5%). Although 1% pirimiphos-methyl produced 100% mortality, it did not exhibit strong or fast-acting knockdown actions which are typical of most organophosphate compounds.

DISCUSSION

Tawi-tawi was the second largest contributor of malaria cases in the Philippines in 2007. In 2013, the province had reported 1,968 confirmed cases, representing 26% of the total number of reported cases in the Philippines (unpublished data, RITM). While *Anopheles flavirostris* is the dominant, or at least putative, malaria vector in most of the country, this study indicted *Anopheles litoralis* is the primary vector species in Tawi-tawi. Thus, continued investigations of the bionomics of this species is needed to determine the appropriateness and effectiveness of ongoing malaria control programs in the Sulu region and if alternative interventions may be applicable to help reduce the risk of malaria transmission further.

Entomological surveys undertaken in TB and SK identified *An. litoralis* as the dominant anopheline encountered among the 11 anopheline species identified in both buffalo (9 spp) and human-baited collections (4 spp) during both collection periods. The HLC results were three times greater during the July than November surveys, a result partly attributed to the presence of inclement weather conditions (typhoon) during November. *Anopheles litoralis* began biting at 06:00 PM and continued throughout the evening into the early morning hours until 06:00 AM when collections ceased. In July, a peak biting time occurred between 10:00 PM to 04:00 AM in TB and 12:00 PM to 02:00 AM in SK while very low numbers were collected in November, therefore precluding an accurate assessment of biting activity that period. No information was obtained whether *An. litoralis* continued biting activity beyond the last hour of collection and it is considered a limitation of the study design.

Based on HLC, *An. litoralis* exhibited greater exophily. The buffalo traps proved productive with 71 and 45.5 mosquitoes collected per animal/night during the first and second surveys, respectively. However, the BBT traps were located nearer breeding habitats compared to the HLC sites giving the BBT a relative advantage. Another study limitation was that the HLC stations in TB were also closer to known productive larval vector habitats compared to SK, thus likely skewing data comparisons between sites.

Indoor adult resting places included walls and under benches and tables. Although slightly exophilic, host-seeking and resting behavior of *An. litoralis* suggested that indoor residual house spraying and the use of insecticide-treated bed nets may be an effective vector control intervention depending on level of community acceptance and utilization. Indoor and outdoor daytime resting harborages were identified and some could be considered as potential targets for focal vector control measures. The tendency of *An. litoralis* to bite humans and other animals, suggests the use of buffalo or other domesticated animals may serve as an diversion and alternative blood source away from humans (termed 'zooprophylaxis') and should be evaluated as a complementary control option. More detailed host range studies would help clarify the extent of biting activity on domestic and wild animals found in the area.

Parous rates varied from July to November, with an apparent older population seen in July. However, the number of mosquitoes examined was relatively small in both surveys, especially in November. Only 19 mosquitoes were dissected for evidence of *Plasmodium* infections, of which 2 specimens (10.5%) were found with midgut infections (oocysts). Salivary gland dissections were negative for sporozoites. There is near certainty that these infections were human plasmodia as non-human primates and other possible sources of *Plasmodium* sporozoites in *Anopheles* (*eg*, *Tragulus* sp/mouse-deer) were absent in the study sites. In future investigations, a more accurate procedure to both detect infections in mosquitoes and identify the parasite species using either a circumsporozoite ELISA or molecular-based PCR method would be advantageous.

Large numbers of *An. litoralis* larvae were found in brackish water swamps in the vicinity of the collection sites. These sites were exposed or partially shaded during the daytime and contained floating natural debris and vegetation (algal mats). Most sites were exposed to direct sunlight during the mid-day hours. The swamp areas found most productive for vector larvae were close to households. Use of environmental management techniques to either modify or eliminate breeding places (*eg*, physical removal of algae, increased tidal flow or tidal flushing) should be taken into consideration as larval source reduction measures on a site-by-site basis. Natural invertebrates and fish predators were commonly encountered during larval collections. The role and impact of predators on larval vector populations requires further study to include identification of predators (and parasites and pathogens) as potential biological control agents for propagation and release against *An. litoralis*.

Measurements made on larval habitats revealed *An. litoralis* is capable of surviving in water with an average dissolved oxygen level of 2.97mg/l, typical of still water systems. A surprisingly low salinity level (0.04%) was seen in habitats sampled but appears sufficient for *An. litoralis* to propagate but more observations are needed to confirm the optimal salinity range for development of this species. *Anopheles sundaicus*, a closely related species to *An. litoralis*, shows very similar, if not identical habitat selection (swamps, abandoned saline fish ponds, etc) wherein more typical salinity levels range closer to 0.4% to 0.7% (Laird, 1988). Mean nitrogen content and alkalinity were 0.003 mg/l and 187.4 mg/l; respectively. In Bongao, *An. litoralis* appears to breed exclusively in brackish water swamp environments; however salinity tolerance and full range of acceptable aquatic habitat types needs clarification. Indiscriminate searches of other potential habitats should be done for the possibility of this species utilizing unique sites and inhabiting atypical locations during periods of stress.

House construction practices on Bongao are regarded basic; generally single room structures built on stilts with wooden walls and floors with galvanized iron roofing. Various kinds of domesticated animals are commonly in close proximity (outdoors or underneath) to the house. Insecticide-treated bed net material used as screens or curtains over open windows and doors may help reduce entry of vectors seeking blood meals or resting sites. Human behavior likely plays a strong role in transmission in Bongao, especially among adult males who are more likely to remain out-of-doors during later evening hours and thus at greater risk of acquiring malaria. The common practice of stores closing around sunset with most households retiring indoors early evening would appear conducive for both IRS and bed nets being beneficial control interventions. Greater health awareness about malaria and dissemination of information on practical personal protection practices at night is recommended in these communities.

Based on WHO insecticide susceptibility tests, the *An. litoralis* populations tested in Bongao were found completely susceptible to the organophosphate, pirimiphos-methyl and the pyrethroid, deltamethrin. There was evidence of possible low-grade resistance to recommended diagnostic concentrations of the other three pyrethroids, permethrin, etofenprox, and lambda-cyhalothrin. Annual or biennial monitoring of susceptibility should be done to determine and ensure sufficient efficacy of insecticides in use. Studies on the residual efficacies of chemicals applied to wall surfaces and bed nets using cone bioassays would also provide important information on the duration of toxic efficacy between spray rounds and the ideal frequency of re-treatments.

There are limitations to the study findings and interpretations. Both survey periods were spatially and temporally limited in scope and therefore may only provide a 'snapshot' of the full range of habitats and behaviors of *An. litoralis* if observed over a longer period of time and more locations. Only a limited number of dissections of *An. litoralis*, 19 of 188 (10%) were conducted to determine malaria infectivity. Neither the circumsporozoite ELISA nor a PCR method was used to either confirm infection or determine the species of parasite. However, as this was by far the most predominant and consistent anopheline collected on human and animal bait, and the dominant species found breeding in and around the two study villages during both surveys, the epidemiological evidence strongly indicates this species to be the most important, if not sole, malaria vector species in the area. This conclusion may hold true throughout much, or all, of the Sulu region. Future studies might also include comparing differences in biology and vectorial capacity of *An. litoralis* found in other locations in the Philippines.

At the time of investigation, the insecticide susceptibility assays indicated that the wide-scale use of insecticide-treated bednets and indoor residual sprays would likely remain beneficial in suppressing indoor malaria transmission on Bongao. Ideally, the determination and confirmation of insecticide susceptibility status would have benefited more had a minimum of 100 female mosquitoes been tested per insecticide. As *An. litoralis* was either found completely susceptible or with evidence of low-grade resistance to several of the pyrethroid compounds used for vector control, additional bioassays are needed to confirm this and also explore the possible metabolic and target site mechanisms involved.

Limited budgets and site remoteness, along with decades of poor civil security in the Sulu area has been one of the most challenging and important limitations for conducting malaria vector investigations in the region. Far more comprehensive and longitudinal studies on the vector ecology, seasonal fluctuations and human social factors influencing malaria transmission will improve knowledge and utilization of confined control program resources. This information could better ensure the most appropriate, efficient, and cost-effective interventions are implemented in line with the local conditions and epidemiology. Bongao Island was also found at risk for dengue transmission as indicated by the common presence of *Aedes aegypti* and *Aedes albopictus*. It would be equally important to define important factors affecting the transmission of this and other mosquito-borne diseases and encompass all in an integrated vector control program.

This vector survey provides valuable information on *An. litoralis* and its potential importance in malaria transmission on Bongao Island. By some measure of careful extrapolation, this species would likely behave much the same throughout all, or much of the Sulu region. In general, published accounts on *An. litoralis* remain woefully limited and there is a need to perform further investigations on the bionomics and epidemiological importance of this species. A better undertanding of the multiple factors in transmision and disease risk would also help direct the most appropriate interventions needed in malaria-affected communities.

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