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Preliminary observations on biology of a man-and cattle-biting *Phlebotomus major major* and a cave dwelling *Phlebotomus stantoni* under laboratory conditions

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Abstract. Increasing evidence of autochtonous leishmaniasis in Thailand has raised concern to understand how transmission of leishmaniasis occurs and determine its epidemiology for disease control. However knowledge of the vectorial capacity for *Leishmania* has been limited by difficulties and failure of sand fly breeding in the laboratory. In this study, a colony of *Phlebotomus major major* and *Phlebotomus stantoni* were established under laboratory conditions. Both colonies were started with a single gravid female and allowed to observe for all developmental stages. We reported their biological characteristics for the first time to be a baseline data for planning vector control measures. The life cycle of *P. major major* and *P. stantoni* are commonly completed in 66 days (range 48-76 days) and 48 days (range 39-49 days), respectively. Eggs hatched within 10 days after being laid. Survival rate of entire life cycle for *P. major major* was 38% and 100% for *P. stantoni*. Our results suggest that attempts to determine biological characteristics of the sand fly species and their vector potential remain vital if special attention is given to successful colonization.

INTRODUCTION

Autochthonous visceral and cutaneous leishmaniasis cases appear to increase in various parts of southern and northern Thailand since 1996 with 12 cases were described as an individual case report by Suankratay (2014). As a consequence of the increase in outbreak occurrence, comprehensive disease investigations in areas of *Leishmania* infection have been conducted for years since then. However supporting evidence for the sand fly species being responsible in *Leishmania* transmission remains obscure. Regarding feeding habits and natural infection, however, four sand fly species including *P. argentipes*, *P. hoepplii*, *P. major major* (Apiwathnasorn *et al*, 1993; 2011) and *Sergentomyia gemmea* (Kanjanopas *et al.*, 2013) are at present potential vectors of leishmaniasis. In addition *L. siamensis* was discovered as a novel species causing autochthonous visceral leishmaniasis in a Thai patient (Sukmee *et al.*, 2008). Pothirat *et al.* (2014) reported first autochthonous case of visceral leishmaniasis caused by *L. martiniquensis* in a 52-year-old Thai male from northern Thailand and later another two *martiniquensis* cases of disseminated cutaneous leishmaniasis associated with HIV infection (Chiewchanvit *et al.*, 2015). An increase in the occurrence of leishmaniasis within a past 5-year period suggests that this infection may be becoming more prevalent than it has been in the previous decades. Regarding incomplete knowledge on *Leishmania* transmission cycle in particular the vector aspect, leishmaniasis control in Thailand is mainly based on its epidemiological features for case detection and treatment. Unfortunately, to date there are no detailed biological data for

any Thai species as a result of failure to establish continuous colonies. Repeated attempts to obtain a permanent colony of the insect vectors have been unsuccessful for reasons as yet unknown. Time to observable impact of the control program will depend in part on the generation time of the target vector species. Regarding feeding behavior, *P. major major* which is anthropophilic in some areas (Apiwathnasorn *et al.*, 2011; Shahar *et al.*, 2011) but zoophilic, feeding preferentially on cattle, in others (Apiwathnasorn *et al.*, 1993; 2011) are collected more frequently by landing collections. In addition, *P. major* s.l. has been reported to be responsible for the transmission of the Sand fly Fever Turkey Virus (Ergunay *et al.*, 2012). The present study demonstrated the biology at various developmental stages of anthropophilic and zoophilic *P. major major* and *P. stantoni* under laboratory conditions in the hope of providing preliminary basic background and data useful for the public health authorities to develop guidelines for entomological surveillance, planning control strategies and monitoring the control durability.

MATERIALS AND METHODS

Attempts to establish sand fly colonies in a paticular genus *Phlebotomus* were initiated from field-collected live specimens by light traps and cow baited traps since 2006. A cow-baited trap located approximately at 100 m distance from Phra Phothisat cave entrance and 4 CDC miniature light traps were placed inside the cave and operated for over a period of three successive nights per month of August-October during 2011 to 2013 in order to collect the target species including *P. argentipes*, *P. major major* and *P. stantoni*. Both blood-engorged and unfed sand flies were kept separately in a screen-topped paper cups provided with 10% sugar solution as energy source and humidity and transported to the insectary. The unfed sand flies were subjected to feed on a hamster which was anesthetized by intraperitoneal injection of 0.35 mg Nembutal per 10 g body weight. The anesthetized hamster was placed on the nylon mesh screen cover, through which the sand flies fed for about 1 h. The engorged females that survived 3 days were individually transferred to lay eggs into an oviposition container (clear plastic container filled with 2 cm of moist plaster of Paris on its bottom). The sand flies that fed on the first blood meal and completed oviposition were subjected to a second blood meal. The dead female was removed from the container to prevent bacterial or fungal contamination. The rearing containers were observed daily to check the mortality of gravid females and the presence of eggs. After laying the eggs, the females were preserved in 90% ethanol for few days and later mounted in Hoyer's medium for species identification following the taxonomic key by Lewis (1978). Although certain species of sand flies had been maintained in many laboratories and a variety of methods used by different workers, our rearing procedures were modified from the techniques described previously by Endris *et al.* (1982). The insectary was set at 26-28ºC with 60% RH and a light/dark regime of 14/10 h supplemented by 60W fluorescent light. The eggs were allowed to hatch in the same containers and the hatched larvae were maintained until emerging to adult. The number of eggs laid and larvae of different stages were counted and recorded. The larvae from the individual batch were reared on a diet consisting of 1:1 mixture of ground dried rabbit feces and rabbit chow enriched with powdered brewer's yeast. The larval food was sprinkled over the eggs on the damp plaster-lined container since newly hatched larvae began feeding immediately. Observations on metamorphosis for developmental durations and mortality of each individual were made and recorded.

RESULTS

Seventeen fully engorged females of *P. major major* were collected by cow-baited nets but most of them died before ovipositing and merely a single live specimen survived through initiating a laboratory colony. Similar to *P. stantoni*, 23 unfed females were obtained from light traps in the cave, three of which received successive blood meals from

anesthetized hamster in the insectary and only one survived through initiating a laboratory colony. A total of 37 *P. argentipes* were collected from the cave but our efforts failed to provide blood-feeding. Oviposition of *P. major major* and *P. stantoni* started 4 days after blood feeding and completed egg-laying by the Day 7. None of sand flies accepted the second blood meal. The successful rearing for one generation permitted us to determine for the first time the duration of every developmental stage of *P. major major* and *P. stantoni* (Table 1 and Table 2). The gravid *P. major major* and *P. stantoni* laid a batch of 29 and 20 eggs, respectively. Hatching rate was lower in *P. major major* accounted for 72.4% than that of *P. stantoni* (100%). Eggs hatched after 10-14 days for *P. major major* and 11-12 days for *P. stantoni*. Both of them completed the life-cycle from egg to adult longer than 40 days. The median development time from egg hatching to adult emergence of *P. major major* was 66 days, with a wide range of duration (48 to 76 days) and the median times required for egg, larval and pupal development were 11, 43 and 12 days, respectively. The median *P. stantoni* egg, larval, and pupal duration were 12, 25 and 11 days, respectively with median development time of 48 days ranging 39-49 days. The percentage of *P. major major* pupae to emerge as adults was 84.6 yielded 4 males and 7 females. In other word, approximately 62% of *P. major major* immatures died before adult emergence. In contrast, the survival rate of *P. stantoni* from egg through adult was 100%.

Table 1. Developmental duration of immature *P. major major* and adult stage under laboratory conditions

Stage	Number	Duration (days)	
		Range	Median
Egg	29	10-14	11
1st stage Larva	21	9-11	9
2nd stage Larva	17	9-12	10
3rd stage Larva	14	11-14	13
4th stage Larva	14	10-13	11
Pupa	13	9-12	12
Adult 11 male 4, female 7			
Egg-Adult		48-76	66

Table 2. Developmental duration of immature *P. stantoni* and adult stage under laboratory conditions

Stage	Number	Duration (days)	
		Range	Median
Egg	20	11-12	12
1st stage Larva	20	6-7	7
2nd stage Larva	20	6-7	7
3rd stage Larva	20	4-5	5
4th stage Larva	20	4-6	6
Pupa	20	8-12	11
Adult 20 (male 8, female 12)			
Egg-Adult		39-49	48

DISCUSSION

Although attempts have been made to colonize sand fly in laboratory for several years, however, the establishment of continuous colonies failed. Of about 700 known sand fly species, less than 60 have been successfully colonized in the laboratory (Volf and Volfova 2011). Nevertheless this was the first attempt to raise *P. major major* and *P. stantoni* under laboratory conditions. An important limitation in this study was the restricted number of sand fly samples to initiate the colonies. Sand flies of particular species are habitat-specific with temporal distribution. For instance, *P. major major* can be obtained only from Chumphon, Kanchanaburi and Saraburi Province (Apiwathnasorn *et al.*, 1993; 2011) and *P. argentipes* is prevalent during July-November (Polseela *et al.*, 2011a; 2011b). Therefore, locality limits the species occurrence, while seasonality limits the length of time that available target samples can be obtained. In addition, the initiating a laboratory colony of sand fly is more difficult than the maintenance of already-established colonies (Killick-Kendrick 1978; Volf and Volfova 2011). *P. major major* and *P. stantoni* could not be colonized through two consecutive generations. The former did not adapt readily to the laboratory culture as the adult emergence rate was lower than 40% in contrast to that of the latter which yielded 100% emergence rate. *P. stantoni* have been reported as a common cavernicolous species in various limestone caves (Apiwathnasorn *et al.*, 1989; 2011; Polseela *et al.*, 2011a) including an edible-nest swiftlet cave of the isolated island (Chittsamart *et al.*, 2015) as well. In addition, *P. stantoni* also inhabited urban environment of Bangkok and Nonthaburi Province (Apiwathnasorn *et al.*, 1989). Probably *P. stantoni* is capable of surviving harsh environments and then more adaptability to laboratory conditions than *P. major major*. Furthermore this species was found to be an anthropophilic species in Gunung Senyum, Malaysia (Khadri Shahar *et al.*, 2011). *P. major major* is of public health importance as a man- and cattle-biting species although it has never been found infected with *Leishmania* parasites in Thailand, yet. It was rare species having been found in 3 locations and biting people outdoors over a short period of time (08:00-11:00 pm) in a low density of few sand flies per person-hour (Apiwathnasorn *et al.*, 1993; Apiwathnasorn *et al.*, 2011). Rare insect species often have subtle habitat requirements (Thomas 1994) and possibly less adaptive potential to the captive or laboratory environment. The ability of sand flies to complete more than one gonotrophic cycle represents an important aspect of their vector potential. This noteworthy aspect remains obscure as both species did not survive second blood feeding. *P. major* and *P. stantoni* deposited less than 30 eggs per batch which were slightly low as female sand flies usually lay 30-70 eggs during a single gonotrophic cycle (Service 2008).

The life cycle of sand flies are varied by species and rearing methods because they require an adaptation to the laboratory environment (Ward 1977) including temperature, humidity and nutrient availability. The life cycle of *P. major major* and *P. stantoni* which last longer than a month appears to be similar to those records of other colonized sand fly vectors. Development time from egg to adult of *P. argentipes* reported by Ghosh and Bhattacharya (1989) was about 32 days at 28-31°C while the development of some Kenyan *Phlebotomus* spp. from egg to adult took 54.7-57.2 days (Mutinga *et al.*, 1989). Numerous difficulties were encountered culturing phlebotomine sand flies. The problems associated with initiation and maintenance of these two species colonies were observed to be death of colonized females at oviposition, larval loses due to fungal contamination and the reluctance of the adult females to feed on the blood sources provided. In the present study, a ground dried rabbit feces and rabbit chow enriched with powdered brewer's yeast was initially used, but it resulted in proliferation of a large amount of fungi and mortality among the different instar larvae of *P. major major*. Marchais *et al.* (1991) indicated that fungal contamination was one of the important difficulties in sand fly rearing. Furthermore

temperature, humidity and larval diet were possibly critical factors when the field collected specimens were brought to an insectary. Hence, it is important to be aware of the basic requirements of different species such as rearing facilities and procedures, environmental controls and nutrition before it could be reared under the laboratory conditions. Nevertheless we were able to complete one successive generation of *P. major major* and *P. stantoni* from eggs.

CONCLUSION

Biological data of vector species will be integrated with epidemiological data to build an entomological surveillance system for leishmaniasis control. Knowledge of the vectorial capacity for *Leishmania* has been limited by difficulties and failure of sand fly breeding in the laboratory. Our potential sand fly vectors have defied all attempts at laboratory cultivation. Where not currently feasible for a particular species to have better understanding of the biological basis, this should be a research priority by investment in scale-up and improved rearing technique. The baseline data of this study could be useful for further attempts to develop a successful rearing technique which will offer sufficient quality materials for determination of vector potential, life-cycle of *Leishmania* and transmission by bite.

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