CHAPTER 1

Introduction

1.1 Statement and Significance of the Problem

Eight species of the Hyrcanus Group (*Anopheles argyropus*, *Anopheles crawfordi*, *Anopheles nigerrimus*, *Anopheles nitidus*, *Anopheles paraliae*, *Anopheles peditaeniatus*, *Anopheles pursati* and *Anopheles sinensis*) found throughout Thailand were recorded also in East, South and other Southeast Asian countries. Some of these species have been incriminated as suspected vectors of malaria due to *Plasmodium vivax*; secondary and potential vectors of filariasis caused by *Wuchereria bancrofti* and *Brugia malayi*, respectively; and secondary vectors of Japanese encephalitis virus. However, very few research experiments concerning these anopheline species have been documented during the past two decades, particularly those with a complete multidisciplinary approach (combined related-aspects of morphology, cytology, molecular investigation, hybridization, susceptibility and refractory to pathogens, etc.). This might result from the lack of biological information and/or available laboratory-raised colonies, particularly adaptive stenogamous colonies that are easy to maintain and mass produce under a standard 30 cubic cm cage, which reduces time, workload and manpower for artificially mating adult females with males.

In natural conditions, anopheline females are mated when entering swarms of males, which usually appear above tops of bushes and other objects. Each female is caught by a male that locates her from a flight tone (mostly within the range of 200-600 Hz), which is proportional to wing size and wing-beat frequency. This flight tone is heard through the hearing organs (Johnston's organ) in the antennae, and after coupling the two mosquitoes fall out from the swarm. Some species (e.g., *Anopheles maculatus*) swarm 15-21 feet from the ground, which is rather high in comparison to other anopheline species.

Nonetheless, success in couples catching each other is based entirely on contact with the sex pheromone of conspecific females and males. Furthermore, detection of this active substance involves a number of olfactory receptors [e.g., single-walled multiporous hair sensilla (sensilla trichodea; A1, A2), single-walled multiporous peg sensilla (sensilla basiconica; capitrate pegs), double-walled multiporous peg sensilla (sensilla basiconica; A3, A4; grooved pegs) and sunken double-wall multiporous peg sensilla (sensilla coeloconica or pitted peg)] located on antennal segments. In laboratory conditions, the limited space in a standard 30 cubic cm cage or other small areas appears to inhibit or reduce the formation of dancing male swarms. Therefore, this causes copulation failure (eurygamous behavior), while many species belonging to the genus *Aedes*, *Culex* and *Mansonia* can copulate without male swarms, and mate easily in small spaces (stenogamous behavior). Of 80 vector-species of anophelines throughout the world, only 20 were reported to copulate in a standard 30 cubic cm cage, i.e., *Anopheles quadrimaculatus*, the Gambiae Complex, *Anopheles earlei*, *An. sinensis*, *Anopheles farauti* No. 1, *Anopheles albimanus*, *Anopheles subpictus*, *Anopheles cracens*, *Anopheles annularis*, *Anopheles dirus*, *Anopheles barberi*, *Anopheles sergentii*, *Anopheles freeborni*, *Anopheles barbirostris*, *Anopheles minimus*, *Anopheles albitarsis*, *An. maculatus*, *Anopheles aquasalis*, *Anopheles stephensi* and *Anopheles pseudopunctipennis*. In addition, evidence of the difference in male genitalia morphometry, frequency of clasper movements and mating time between stenogamous *An. cracens* and eurygamous *An. dirus* have been documented. The genitalia of *An. cracens* are larger than that of *An. dirus* and the former has a shorter duration of clasper movement and mating time than the latter species.

As pointed out by the above information, there is a shortage of knowledge on the stenogamous/eurygamous behavior of eight species of Hyrcanus Group, except for *An. sinensis* (Korean strain). This study proposes to establish adaptive stenogamous colonies of eight species of Hyrcanus Group and trial to search for possible mechanism(s) that support stenogamous behavior. Therefore, this study made detailed investigation by comparing: (1) the mating ability of adult mosquitoes in a 10, 20, 30 and 40 cubic cm cage at two density resting surface (DRS) of 3.6 and 7.2; (2) the measurements of male and female wings, female maxillary palpomeres and male genitalia; (3) the number of large sensilla coeloconica on the antennae of females; and

(4) the frequency of clasper movement in male genitalia during induced copulation, and duration of mating between stenogamous and eurygamous species.

1.2 Literature Review

1.2.1 Distributions and medical important

The Hyrcanus Group (Genus *Anopheles*, Subgenus *Anopheles*) is distributed widely from Europe to East and Southeast Asia, including some of the off-lying islands of the Indian and Pacific Oceans, and at least 27 species are reported within it (Reid 1968; Paredes-Esquivel et al. 2011; Harbach 2015). In Thailand, eight species of the Hyrcanus Group have been reported so far, i.e., *An. argyropus*, *An. crawfordi*, *An. nigerrimus, An. nitidus, An. paraliae* (= *Anopheles lesteri*, Taai et al. 2013), *An. peditaeniatus*, *An. pursati* and *An. sinensis* (Harrison and Scanlon 1975; Rattanarithikul et al. 2006). Among these, *An. peditaeniatus* and *An. sinensis* are considered as suspected vectors of malaria due to *P. vivax* in Thailand (Baker et al. 1987; Harbach et al. 1987; Gingrich et al. 1990; Frances et al. 1996; Rattanarithikul et al. 1996), while they have been incriminated as natural vectors of *P. vivax* in China and Korea (Liu 1990; Lee et al. 2007) and Japanese encephalitis virus in China and India (Mourya et al. 1989; Zhang 1990; Kanojia et al. 2003), respectively. Although *An. peditaeniatus* has been found abundantly and widely distributed throughout Thailand (Harrison and Scanlon 1975; Scanlon et al. 1968), its status as a vector of the Japanese encephalitis virus is still a cryptic question, which needs to be investigated more intensively. Recently, *An. sinensis* and *An. nigerrimus* have been incriminated as a main vector and secondary or incidental vector, respectively, of *W. bancrofti* in Asia (Manguin et al. 2010). In addition, *An. peditaeniatus*, *An. crawfordi*, *An. nigerrimus*, *An. argyropus* and *An. pursati* were reported as high potential vectors of nocturnally subperiodic *B. malayi* (Saeung et al. 2013). Likewise, the Hyrcanus Group was also considered as an economic pest of cattle because of its vicious biting-behavior and ability to transmit cervid filariae of the genus *Setaria* (Reid 1968; Harrison and Scanlon 1975).

1.2.2 Mating behavior

The mating behavior of anophelines in nature, emerged adults from pupae seek mating at the first opportunity, which is usually in the first evening. Males form in dancing swarms at dusk, usually above bushes and other objects, whereas females may be seen to enter these swarms in small numbers (Reid 1968). Observation of swarming *Anopheles philippinensis*, *Anopheles indiensis*, *Anopheles vagus*, *Anopheles kochi* and *An. maculatus* indicated that swarms of *An. maculatus* were 15-21 feet from the ground, which was rather high in comparison to other anopheline species (Wharton 1953). Additional mating behavior results in nature of *An. stephensi* var. *mysorensis* revealed approximately 400 copulations per swarm of 500-600 males, thus, confirming that anopheline females are mated by entering swarms of males (Quraishi 1965). Males within dancing swamps give a mating response when stimulated by flight tones (wing-beat sound) of a conspecific female flying nearby. Sound generated by the beating of female wings consists of a harmonious series that provides most acoustic energy (mostly within the range 200-600 Hz), which is proportional to wing size, wing-beat frequency and ambient temperature (Belton and Costello 1979; Tamarina et al. 1980; Brogdon 1994; Wekesa et al. 1998). Each female is grabbed promptly by a conspecific male, which locates her through the hearing organs (Johnston's organ) in his antennae, and then the couple can be seen to fall out from the swarm (Tamarina et al. 1980). Analysis of the interaction between free-flying males and tethered females of *An. gambiae* s.s., which were able to beat their wings and move their legs, revealed the following sequence of activities (Figure 1.1). (1) a male approached a flying female, and hooked the large tarsal claw on one of his forelegs on to one of the female's legs, usually a foreleg, (2) the male swung himself under the female, quickly bent his abdomen upwards and brought his genitalia in contact with those of female as venter-to-venter position, (3) having interlocked his genitalia with those of female, the male released his hold on the female's legs and adopted an end-to-end position, and (4) after 13-14 seconds the male oscillated vigorously from side to side and then, after a further 2-3 seconds, opened his clasper and flew away. The mean duration of copulation was 17 seconds. During the early stages of copulation, flight was inhibited, but once in the end-to-end position both partners resumed flight. Removal of the males' foretarsal claws in most cases prevented coupling (Charlwood and Jones 1979).

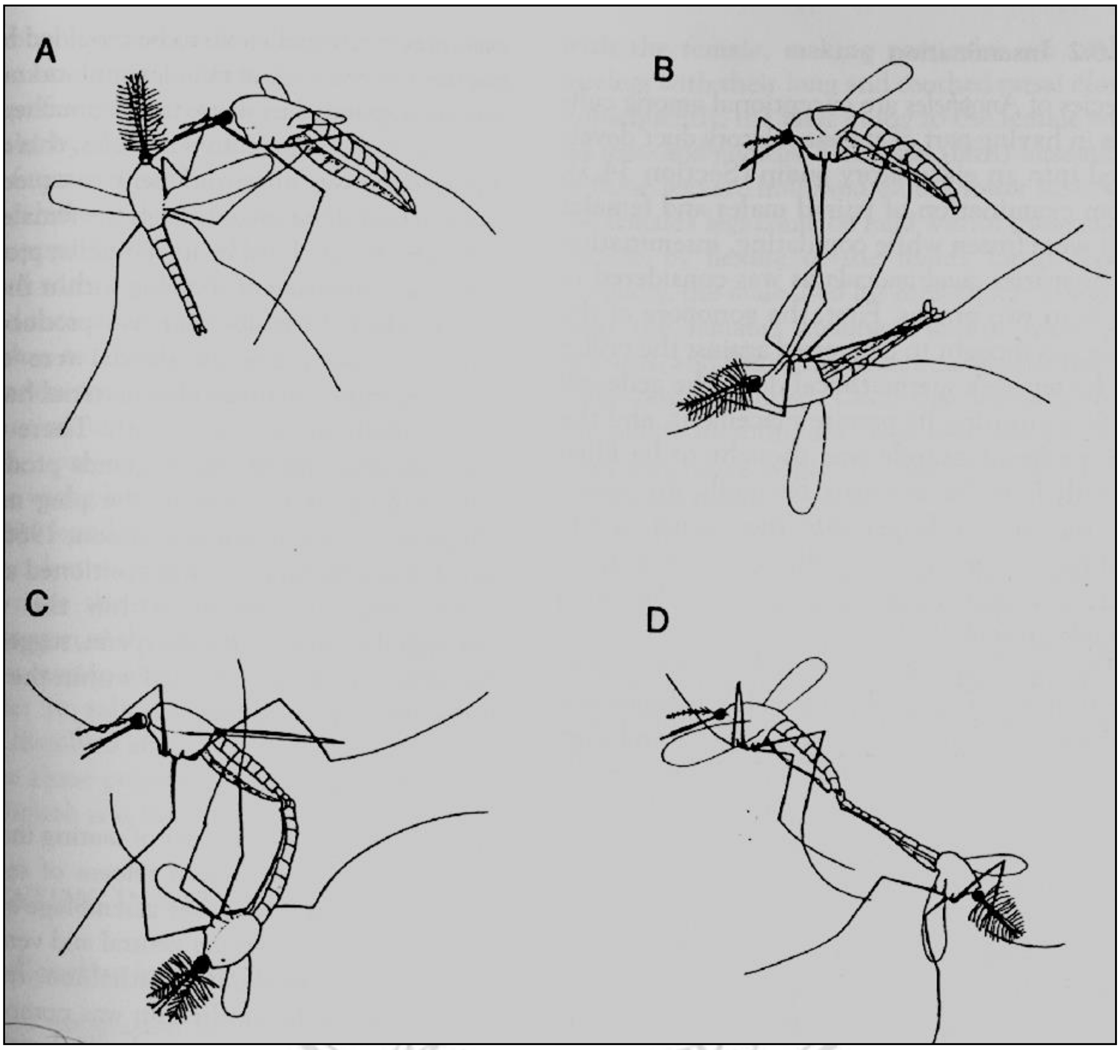

Figure 1.1 Sequences in the coupling of *Anopheles gambiae* s.s., drawn from photographs of males coupling with females suspended from thorax (Charlwood and Jones 1979)

Besides the above systems, particularly the wing-beat frequencies, the involvement of sex pheromone in species recognition of conspecific females and males has been documented as an important factor. This active substance was non-volatile, heat-stable, and soluble in solvents of intermediate polarity. It remained active for 2-3 weeks after the death of a female (Lang and Foster 1976; Lang 1977). Attempts by caged males of *Mansonia uniformis* and *Mansonia africana* to copulate with dead females were suggestive of a contact sex pheromone (Laurence 1960). Furthermore, the detection of this active substance involved a numbers of olfactory receptors, i.e., single-walled multiporous hair sensilla (sensilla trichodea; A1, A2), single-walled multiporous peg sensilla (sensilla basiconica; capitrate pegs), double-walled multiporous peg sensilla (sensilla basiconica; A3, A4; grooved pegs) and sunken double-wall multriporous peg sensilla (sensilla coeloconica or pitted peg) (Clements 1999; Pitts and Zwiebel 2006).

Additional supportive experiments were observed clearly between *Aedes aegypti* and *Aedes albopictus*. When males of *Ae. albopictus* or *Ae. aegypti* were caged with virgin females of their own and another *Aedes* species, males of *Ae. albopictus* invariable coupled only with females of their own species, whereas males of *Ae. aegypti* were less discriminatory. In one experiment procedure, a female of *Ae. albopictus* was suspended by the thorax in a cage containing males of the same species. When the female beat her wings, males flew towards her and one copulated with her. When a female of *Ae. aegypti* was suspended in the cage with males of *Ae. albopictus*, the males approached her as soon as she beat her wings, but immediately they had touched her, they flew off and made no further approaches. When a female *Ae. albopictus* with wings glued together was suspended in the cage, in close contact with the screen, the males ignored her. However, when a female *Ae. aegypti* suspended outside the cage, but immediately over the *Ae. albopictus* female, beat her wings, the male were immediately attracted to the immobilized *Ae. albopictus* female and copulation ensued. The males had oriented towards the flight sound of the heterospecific female. When the positions of the females were reversed, an immobilized *Ae. aegypti* being placed inside the cage and an *Ae. albopictus* outside, the male of *Ae. albopictus* flew to the *Ae. aegypti* female on hearing the sound stimulus but flow off after touching her. Thus, the *Ae. albopictus* males responded to the flight tone of a female of either species, but showed species discrimination only after making contact with her (Nasci et al. 1989; Black et al. 1989; Duhrkopf and Hartberg 1992).

Anopheline mosquitoes have difficulty or failure in copulating naturally under laboratory conditions, especially in a small-sized cage (eurygamous behavior) such as a 30 cubic cm cage, which appeared to inhibit or reduce the formation of dancing male swarms. On the other hand, many male species of *Aedes*, *Culex* and *Mansonia* mosquitoes could copulate without forming a swarm, and they mated easily in small spaces (stenogamous behavior) (Wharton 1953; Sasa et al. 1967; Clements 1999).

Therefore, artificial mating techniques have been developed by previous investigators in order to solve the mating problems in maintaining laboratory colonies of other anopheline species (Baker et al. 1962; Ow Yang et al. 1963). It has been documented that the behavioral polymorphism stenogamy/eurygamy of anophelines is inherited and obviously controlled by one or more genes located on the Y-chromosome (Fraccaro et al. 1977).

1.3 Purpose of This Study

1.3.1 To screen the stenogamous behavior of *An. argyropus*, *An. crawfordi*, *An. nigerrimus*, *An. nitidus*, *An. paraliae*, *An. peditaeniatus*, *An. pursati* and *An. sinensis*.

1.3.2 To establish self-mating colonies of species proven to have stenogamous behavior.

1.3.3 To search for possible mechanism(s) that control stenogamous behavior.

1.4 Usefulness of the Study

Success in establishing self-mating colonies of the Hyrcanus Group (*An. argyropus*, *An. crawfordi*, *An. nigerrimus*, *An. nitidus*, *An. paraliae*, *An. peditaeniatus*, *An. pursati* and *An. sinensis*) for many consecutive generations will bring about mass production of these anopheline species, which will reduce time, workload and manpower. Massive colonies of each species will act as main keys for operating various research aspects of Hyrcanus Group-parasite systems in Thailand, as well as South, East and other Southeast Asian countries, in which these anopheline mosquito species members are distributed.