รายงานวิจัยฉบับสมบูรณ์

โครงการ

สารกระตุ้นการเปลี่ยนแปลงทางพฤติกรรมและชีวสภาพของยุงก้นปล่องมินิมัส พาหะนำโรคมาลาเรียในประเทศไทย

BEHAVIORAL MODIFYING COMPOUNDS AND SOME BIONOMICS OF *ANOPHELES MINIMUS* COMPLEX, VECTOR OF MALARIA IN THAILAND

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30 กรกฎาคม 2551

สัญญาเลขที่ RMU 4880032

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โครงการ
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สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษา
และสำนักงานกองทุนสนับสนุนการวิจัย

กิตติกรรมประกาศ

การวิจัยครั้งนี้สำเร็จลุล่วงไปด้วยดีด้วยความร่วมมือของหลายฝ่าย โดยเฉพาะสำนักงานกองทุนสนับสนุนการวิจัย (สกว.) ที่ได้สนับสนุนเงินทุนในการทำการวิจัยโครงการฯ ตามโครงการเลขที่ RMU 4880032 โดยมีนายธีรภาพ เจริญวิริยะภาพ เป็นหัวหน้าโครงการ ซึ่งผู้วิจัยขอขอบคุณมา ณ โอกาสนี้ ขอขอบคุณกองการเกษตรและสหกรณ์ สำนักงานทหารพัฒนา กองบัญชาการทหารสูงสุด บ้านพุเตย ตำบลท่าเสา อำเภอไทรโยค จังหวัดกาญจนบุรี ที่อำนวยความสะดวกสถานที่ในการทำวิจัยภาคสนาม ขอขอบคุณสถาบันวิจัยและพัฒนาแห่งมหาวิทยาลัยเกษตรศาสตร์ ที่ได้ร่วมสนับสนุนสมทบค่าใช้จ่ายในการทำวิจัยเพื่อให้โครงการตามสัญญาเลขที่ RMU 4880032 ดำเนินไปด้วยความราบรื่น สุดท้ายนี้ผู้วิจัยขอขอบคุณ นิสิตและผู้ร่วมงานทุกคนที่มีส่วนร่วมให้งานวิจัยครั้งนี้สำเร็จไปได้ด้วยดี

Abstract

Chemicals protect humans from the bite of insects using three different actions: irritation after making contact, repelling prior to contact, or by killing the insects (toxicity). Most research has focused on the toxic function of chemicals whereas comparatively few have concentrated on non-toxic chemical characteristics. In this study, we tested three actions of test chemicals on *Anopheles minimus* populations using both laboratory and field assay systems. Laboratory investigation was conducted using the free choice excito-repellency (ER) test box. Field studies were performed using experimental huts. Our findings indicate that test chemicals successfully repelled mosquitoes from treated surfaces at low concentrations whereas higher doses provided a toxic action on mosquito populations. In particular, DDT demonstrated a unique property of "repellency" whereas synthetic pyrethroids i.e., deltamethrin primarily functioned as "irritants". The toxic action of chemicals at higher doses could stimulate selection for resistance in vector populations, whereas applying chemicals at minimal doses can help delay physiological resistance and possibly reduce the environmental risk. With the new ER test system, we were also able to test the effect of several pyrethroids on several *Aedes aegypti* strains. In addition, we took the advantages of the PCR technology to identify the two species of *An. minimus* complex. Biting pattern and seasonal abundance between the two species were also characterized.

Key Words: Chemical, irritancy, repellency, DDT, pyrethroids, mosquito

บทคัดย่อ

สารเคมีสามารถใช้ป้องกันยุงไม่ให้กัดคนได้โดยอาศัย 3 กลไก ดังนี้ ทำให้ยุงเกิดความระคายเคือง ไล่ และ ฆ่า งานวิจัยทางด้านกีฏวิทยาทางการแพทย์ในการควบคุมยุงมักสนใจในความเป็นพิษของสารเคมีที่มีต่อยุงเท่านั้น มีงานวิจัยจำนวนน้อยที่ศึกษาประสิทธิภาพการไล่และการทำให้ยุงเกิดการระคายเคืองต่อสารเคมี การศึกษาครั้งนี้ได้ศึกษาฤทธิ์ในการไล่ยุงก้นปล่องมินิมัสจากสารเคมีหลายชนิดโดยเฉพาะสารในกลุ่มดีดีทีและไพรีทรอยด์โดยอาศัยเครื่องมือทดสอบการไล่ติดตั้งคอมพิวเตอร์อัตโนมัติพร้อมทั้งระบุกลไกในการไล่ เครื่องมือนี้ได้พัฒนามาจากการใช้ศึกษากลไกการไล่ของสารเคมีแบบธรรมดา จากการศึกษาพบว่าสารเคมีหลายชนิดมีฤทธิ์ในการไล่ยุงที่ความเข้มข้นต่ำๆ ในขณะที่ความเข้มข้นสูงมักจะฆ่ายุงให้ตาย ดีดีที (standard compound) มีฤทธิ์ในการไล่ที่ดีมากในขณะที่สารเคมีในกลุ่มไพรีทรอยด์มีฤทธิ์ทำให้ยุงเกิดการระคายเคือง การใช้สารเคมีในปริมาณต่ำและเหมาะสมตามประสิทธิภาพของสาว สามารถช่วยชลอการต้านทานสารเคมีในยุงให้ช้าลง นอกจากนี้ได้นำเครื่องมือทดสอบการไล่แบบอัตโนมัติพร้อมทั้งระบุกลไกในการไล่ไปใช้กับยุงลายและยุงรำคาญทั้งในห้องปฏิบัติการและภาคสนาม พบว่าให้ผลใกล้เคียงกับการศึกษาจากยุงก้นปล่อง การใช้กระท่อมทดลองเพื่อศึกษาฤทธิ์ของสารเคมีกับยุงลายและยุงรำคาญพบว่าให้ผลใกล้เคียงกับการศึกษาในห้องปฏิบัติการเช่นกัน ดังนั้นการใช้สารเคมีเพื่อควบคุมยุงจำเป็นอย่างยิ่งที่ควรทราบกลไกของสารเคมีแต่ละชนิดที่ใช้ในการควบคุม ในการศึกษาครั้งนี้ได้นำเทคนิคด้านโมเลกุลมาใช้ในการจำแนกชนิดของยุงมินิมัสชนิดซับซ้อนและได้รูปแบบและช่วงเวลาการเข้าหาเหยื่อของยุงมินิมัสทั้งสองกลุ่ม

คำสำคัญ: สารเคมี, การระคายเคือง, การไล่, ดีดีที, ไพรีทรอยด์, ยุง

Project Code:

RMU 4880032

Project Title:

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BEHAVIORAL MODIFYING COMPOUNDS AND SOME BIONOMICS OF *ANOPHELES MINIMUS* COMPLEX, VECTOR OF MALARIA IN THAILAND

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Executive summary

สารเคมีทีใช้ในการควบคุมแมลงมีคุณสมบัติสามอย่างคือ ฆ่าแมลง (toxicity) ก่อให้แมลงระคายเคือง (irritancy) และไล่แมลง (repellency) คุณสมบัติของสารเคมี มีความสำคัญที่ควรศึกษาให้เข้าใจเพราะการใช้สารเคมีมีผลโดยตรงต่อมนุษย์ สิ่งแวดล้อมและประชากรยุงในธรรมชาติ การสร้างความต้านทานสารเคมีของยุงทำให้การควบคุมด้อยประสิทธิภาพและสิ้นเปลืองเวลาและงบประมาณโดยใช่เหตุ ขณะนี้กลไกของสารเคมีที่มีต่อยุงในเชิงพฤติกรรมมีศึกษากันน้อยมาก นั่นคือการต้านทานสารเคมีโดยการกระตุ้นให้เกิดพฤติกรรมหนีจากสารเคมีหรือการใช้สารเคมีเปลี่ยนแปลงพฤติกรรม หรือที่เรียกกันว่า “PESTICIDE AVOIDANCE” ซึ่งปรากฏการณ์ที่เกิดขึ้น ยังไม่เป็นที่แน่ชัดว่าเกิดขึ้นเนื่องจากสาเหตุใด การไม่มีเครื่องมือหรืออุปกรณ์ที่มีประสิทธิภาพในทดสอบหรือ.ใช้ปฏิบัติงานอาจเป็นสาเหตุหนึ่ง

วัตถุประสงค์หลักของงานวิจัยชิ้นนี้ เพื่อพัฒนาเครื่องมือศึกษากลไกของสารเคมีเพื่อให้เหมาะสมในการไปใช้มากขึ้นทั้งในภาคสนามและห้องปฏิบัติการ นอกจากนี้นำไปใช้ในการตรวจสอบคุณสมบัติเบื้องต้นของสารเคมีและวัดระดับและประเภทของ การต้านทานสารเคมีเชิงพฤติกรรมของพาหะนำโรค โดยเฉพาะยุงก้นปล่องมินิมัสซี นอกจากนี้ได้ศึกษาชีวสภาพทางประชากรของยุงก้นปล่องมินิมัสซี โดยการศึกษาโครงสร้างประชากรและพฤติกรรมต่างๆ เช่น พฤติกรรมการดูดเลือด พฤติกรรมการชอบเข้าบ้าน พันธุศาสตร์เชิงประชากร เป็นต้น

ผลการศึกษาครั้งนี้ เครื่องมือศึกษากลไกสารเคมีที่มีต่อยุงได้พัฒนาขึ้น โดยอาศัยระบบอิเลคโทรนิคคอมพิวเตอร์แทนการใช้คนทำงาน ซึ่งทำให้ได้ผลการทดลองที่ถูกต้องและชัดเจนมากขึ้น ได้ทดลองใช้ทั้งในภาคสนามและห้องปฏิบัติการกับยุงก้นปล่องมินิมัสซี (*Anopheles minimus* C)[1] และยุงลาย (*Aedes aegypti*) โดยใช้สารเคมีในกลุ่มไพรีทรอยด์เป็นต้นแบบ (กระทรวงสาธารณสุขใช้ในการควบคุมแมลง) ในภาคสนามได้นำกระท่อมทดลองเข้ามาใช้ประเมินผลกระทบหลังจากพ่นสารเคมีเหล่านี้เพื่อควบคุมยุงพาหะ นอกจากนี้ได้ศึกษาชีวนิสัยและตรวจสอบชนิดของมินิมัสซี ซึ่งการตรวจสอบชนิดมินิมัสซีได้ศึกษาจากรูปร่างปีก (Wing pattern) และดี เอ็น เอ โดยเทคนิคทางโมเลกุล (Multiplex PCR) และศึกษาความสัมพันธ์ทาง

[1] ปัจจุบันคือ *Anopheles harrisoni*

พันธุศาสตร์ระหว่างประชากรยุงมินิมัสในเขตจังหวัดกาญจนบุรีโดยอาศัยเทคนิดการวิเคราะห์โปรตีน

เนื้อหางานวิจัย

TASK 1. การศึกษาสารกระตุ้นพฤติกรรมของยุงก้นปล่องมินิมัสซี

To develop a more advance repellent test system to use for chemical modifying compounds,

To test known promising compounds used in vector control in order to standardize the new test method, **(ดูเอกสารแนบ** 4)

To evaluate mosquito responses to insecticides by testing irritancy and repellency actions using the excito repellency test system **(ดูเอกสารแนบ** 2, 7, 8)

TASK 2. การใช้กระท่อมทดลองศึกษาในภาคสนาม

To observe to biting activity of *Anophles minimus* into the experimental hut treated with either DDT or deltamethrin **(ดูเอกสารแนบ** 3, 10, 14)

TASK 3 การศึกษาชีวสภาพและประชากรของยุงก้นปล่องมินิมัส

To identify the two species in *Anopheles minimus* complex by morphological and molecular biology **(ดูเอกสารแนบ** 1)

To evaluate feeding behavior and biting cycle **(ดูเอกสารแนบ** 5)

TASK 4. การศึกษาโครงสร้างทางประชากรของยุงก้นปล่องมินิมัสชนิดซับซ้อนในจังหวัดกาญจนบุรี

To determine the genetic variations between *Anopheles minimus* collections **(ดูเอกสารแนบ** 8)

TASK 5. การใช้ GIS ในการศึกษาแหล่งเพาะพันธุ์ของยุงก้นปล่องมินิมัส

To characterize the breeding habitats between the two species, *Anopheles minimus* A (*Anopheles minimus*) and *Anopheles minimus* C (*An. harrisoni*) **(ดูเอกสารแนบ** 13)

TASK 6. การศึกษาสารกระตุ้นพฤติกรรมของยุงอื่นๆ เช่นยุงลาย

To characterize the behavioral responses of *Aedes aegypti* to catnip oil using an automate ER system **(ดูเอกสารแนบ** 12)

บทนำ วิธีการทดลอง ผลการทดลอง บทวิจารณ์ อ้างอิง (ดูเอกสารแนบ)

Output

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

YEAR 2006

1. Sungvornyothin S, Garros C, **Chareonviriyaphap T**, Manguin S. 2006. How reliable is the humeral pale spot for the identification of the two species of the *minimus* complex. ***J. Amer. Mosq. Control Assoc.*** 22: 2 185-191. (เอกสารแนบ 1)

2. **Chareonviriyaphap T**, Kongmee M, Bangs MJ, Satantripop S, Meunworn V, Parbaripai A, Suwondkerd W, Akratanakul P. 2006. Influence of nutritional and physiological status on behavioral responses of *Aedes aegypti* to deltamethrin and cypermethrin. ***J. Vector Eco.*** 31: 1 89-101. (เอกสารแนบ 2)

3. Suwondkerd W, Mongkalakul P, Achee N, Grieco J, Robert F, Roberts DR., **Chareonviriyaphap T**. 2006. The effect of host types on normal movement patterns of *Aedes aegypti* (Diptera:Culicidae) into and out of the experimental hut in Thailand. ***J. Vec. Ecol.*** 31: 311-318. (เอกสารแนบ 3)

4. Tanasinchayakul S, Polsomboon S, Parbaripai A, **Chareonviriyaphap T**. 2006. An automated, field-compatible device for excito-repellency testing in mosquito vectors. ***J. Vec. Ecol.*** 31: 210-212. (เอกสารแนบ 4)

5. Sungvornyothin S, Meunworn V, Garros C, Manguin S, Prabaripai A, **Chareonviriyaphap T**. 2006. Trophic activity of two sibling species of the *Anopheles minimus* complex, a vector of malaria in western Thailand. ***J. Vec. Ecol.*** 31: 252-261. (เอกสารแนบ 5)

6. Maunvorn V, Akratanakul P, Bang MJ, Parbaripai A, **Chareonviriyaphap T**. 2006. Insecticide induced behavioral responses in two populations of *Anopheles maculatus* and *Anopheles swadwongporni*, malaria vectors in Thailand. ***J. Amer. Mosq. Control Assoc***. 22: 689-698. (เอกสารแนบ 6)

YEAR 2007

1. Pothikasikorn J, Overgaads H, Bangs MJ, Chareonviriyaphap T. 2007. Behavioral responses of *Anopheles minimus*, a vector of malaria in Thailand, to agrochemicals ***J. Med. Entomol.*** 44:1032-1039. (เอกสารแนบ 7)

YEAR 2008

1. Polsomboon S, Tanasinchayakul S, Bangs MJ, Poolprasert P, Chareonviriyaphap T. 2007. Effects of physiological conditioning on behavioral avoidance by using a single age group of *Aedes aegypti* expose to deltamethrin and DDT. ***J. Med. Entomol.*** 45: 251-259. (เอกสารแนบ 8)

2. Poolprasert P, Manguin S, Bangs MJ, Sukhontabhirom S, Akratanakul P, Chareonviriyaphap T. 2008 Genetic structure and gene flow of *Anopheles minimus* and *Anopheles harrisoni* in Kanchanaburi Province, Thailand. ***J. Vec. Ecol.*** 33: 158-165. (เอกสารแนบ 9)

3. Polsomboon S, Poolprasert P, Suwonkerd W, Bangs MJ, Tanasinchayakul S, Akratanakul P, Chareonviriyaphap T. 2008. Biting patterns of *Anopheles dirus* complex (Diptera: Culicidae) in experimental huts treated with DDT and deltamethrin. ***J. Vector Ecol*** 33 (2): (in press) (เอกสารแนบ 10)

4. Thanispong K, Sathantriphop, Chareonviriyaphap T. 2008. Insecticide resistance of *Aedes aegypti* (Linnaeus) and *Culex quinquefasciatus* Say in Thailand. ***J. Pest Science*** (in press) (เอกสารแนบ 11)

5. Polsomboon S, Greico JP, Chauhan KR, Tanasinchayakul S, Chareonviriyaphap T. 2008. Behavioral responses of Catnip (*Nepeta cataria* L) by two species of mosquitoes, *Aedes aegypti* (L) and *Anopheles harrisoni* Harbach and Manguin, in Thailand. ***J. Amer. Mosquito Control Assoc.*** (in press) (เอกสารแนบ 12)

2. การนำผลงานวิจัยไปใช้ประโยชน์

การศึกษาสารเคมีที่ใช้ควบคุมยุงในประเทศไทย: งานวิจัยชิ้นนี้สนับสนุนงานวิชาการด้านการควบคุมแมลงพาหะนำโรคของกระทรวงสาธารณสุข ซึ่งกระทรวงฯ มีนโยบายให้มีการใช้สารเคมีควบคุมยุงพาหะนำโรคหลังจากได้มีการทดสอบภายในประเทศแล้ว ผู้วิจัยได้ศึกษากลไกสารเคมีที่ใช้และกำลังใช้ในการควบคุมแมลงพาหะนำโรคครบทั้ง 3 แบบ และได้เสนอข้อมูลที่เป็นประโยชน์เพื่อการพิจารณาการเลือกใช้สารเคมีเพื่อควบคุมโรค ของกระทรวงสาธารณสุข

3. การเสนอผลงานในการประชุมวิชาการนานาชาติ

YEAR 2006

1. **Chareonviriyaphap T,** Sungvornyothin S, Suwondkerd W, Manguin S, Garros C. 2006. Trophic behavior and biting cycles of two species in the Minimus Group. American Society of Tropical Medicine and Hygiene Annual Meeting 55th. 11-16 November 2006. Atlanta. GA, U.S.A (Poster presentation).

YEAR 2007

1. **Chareonviriyaphap T,** Polsomboon S, Poolprasert P, Grieco JP, Achee NL, Bangs MJ, Suwonkerd W. 2007. Movement pattern of *Anopheles dirus* into the experimental huts treated with DDT and deltamethrin. American Society of Tropical Medicine and Hygiene Annual Meeting 56th. 4-8 November 2007. Philadelphia, PA. U.S.A. (Poster presentation).

YEAR 2008

1.**Chareonviriyaphap T,** Grieco J, Achee N, Suwondkerd W, Monklangkool P. 2008. Invited speaker in the Symposium entitled *"pesticide and environment"* in the 74thAnnual Conference of the American Mosquito Control Association, Reno, Navada, USA. 2-6 March 2008 (Oral Presentation).

2.**Chareonviriyaphap T,** Poolprasert P, Suwondkerd W. 2008. Biting pattern of *Anopheles minimus* into the experimental hut treated with DDT or Deltamethrin. XXIII International Congress of Entomology 6-12 July 2008. International Convention Center, Durban, South Africa (Oral Presentation).

ภาคผนวก (reprints and manuscripts) (แนบ)

Journal of the American Mosquito Control Association, 22(2):185–191, 2006

HOW RELIABLE IS THE HUMERAL PALE SPOT FOR IDENTIFICATION OF CRYPTIC SPECIES OF THE MINIMUS COMPLEX?

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ABSTRACT. The *Anopheles minimus* Complex Theobald (Diptera: Culicidae) is composed of the 3 sibling species A, C, and E. The malaria vectors *An. minimus* A and C are distributed over the Southeast Asian region, whereas species E is restricted to the Ryukyu Japanese islands. Because species A and C can be sympatric and present specific behaviors and have a role in malaria transmission, it is important to differentiate them. The literature mentioned the presence of a presector pale spot on the wing costa of *An. minimus* A, whereas species C may exhibit both presector and humeral pale spots. However, the reliability of their diagnostic power has not been established over large temporal and geographic surveys. From the analyses of 9 populations throughout Southeast Asia, including published data and field populations from 2 sites in Thailand, we showed that the wing patterns present spatial and temporal variations that make these two morphological characters unreliable for the precise identification of *An. minimus* A and C. Therefore, molecular identification remains the most efficient method to obtain an unambiguous differentiation of these 2 species. Correct species identification is essential and mandatory for any relevant study on the Minimus Complex and for the application of successful control strategies.

KEY WORDS *Anopheles minimus* Complex, morphological identification, diagnostic character, malaria, Asia

INTRODUCTION

Anopheles minimus Theobald was described in 1901, and currently the Minimus Complex is composed of the 3 sibling species A, C, and E (Harbach 1994, 2004; Somboon et al. 2001). *Anopheles minimus* species A and C are widespread over the Asian continent (Green et al. 1990, Van Bortel et al. 1999, Chen et al. 2002) and can be sympatric, whereas species E is restricted to the Ryukyu islands in Japan (Somboon et al. 2001, 2005), a malaria-free region. By definition, no morphological characters exist that could clearly identify the 3 species. However, Sucharit et al. (1988) presented a potential diagnostic character that could differentiate the two species. *Anopheles minimus* A may present a wing costa with a presector pale spot (PSP phenotype), whereas *An. minimus* C may exhibit both presector pale and humeral pale spots (HP phenotype) (Fig. 1). *Anopheles minimus* E seems to be distinct from species A and C (presence of both a humeral pale spot and a pale fringe spot at the tip of vein 1A), although there is no unique character or set of characters that are peculiar to it (Somboon et al. 2001). Evidence of morphological differences between eggs of species A and C also was reported (Sucharit et al. 1995), but only colony populations were tested, which raises the question of the validity of this character in natural populations.

Several studies used the two phenotypes to identify both species (Green et al. 1990, Van Bortel et al. 1999, Chen et al. 2002). Moreover, this potential diagnostic character is routinely used in Asia during entomological field surveys when molecular identification is not feasible (Rwegoshora et al. 2002). Recently, several molecular assays were developed to facilitate the identification of both sympatric species (Sharpe et al. 1999; Van Bortel et al. 2000; Phuc et al. 2003; Garros et al. 2004a, 2004b). The reliability of the humeral spot diagnostic power being little tested over large temporal and geographic surveys, we conducted a study to assess and compare the polymorphism of this character over 9 wild populations throughout Southeast Asia. The aim of the present work was to define whether a morphological identification of *An. minimus* A or C based on these characters is reliable.

Several previous studies included both morphological and other identifications (isoenzymes or DNA-based assays). Green et al. (1990) scored females from western Thailand (Kanchanaburi Province) for the presence or absence of the humeral pale spot and compared the identifications with isozyme assays. These authors found that the majority of *An. minimus* species C had the HP phenotype and that this character may differentiate the 2 species with an error of 37%. In Japan, Somboon et al. (2001) followed the morphological variations of *An. minimus* E during 1 year considering separated males and females. He concluded that seasonal variations existed with a decrease of the presence of the pale spots during the winter. Variations were independent of sex. In northern Vietnam, Van Bortel et al. (1999) evaluated the diagnostic power of

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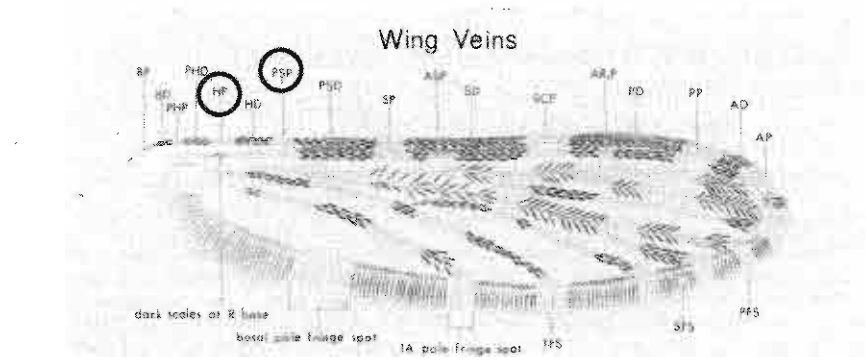

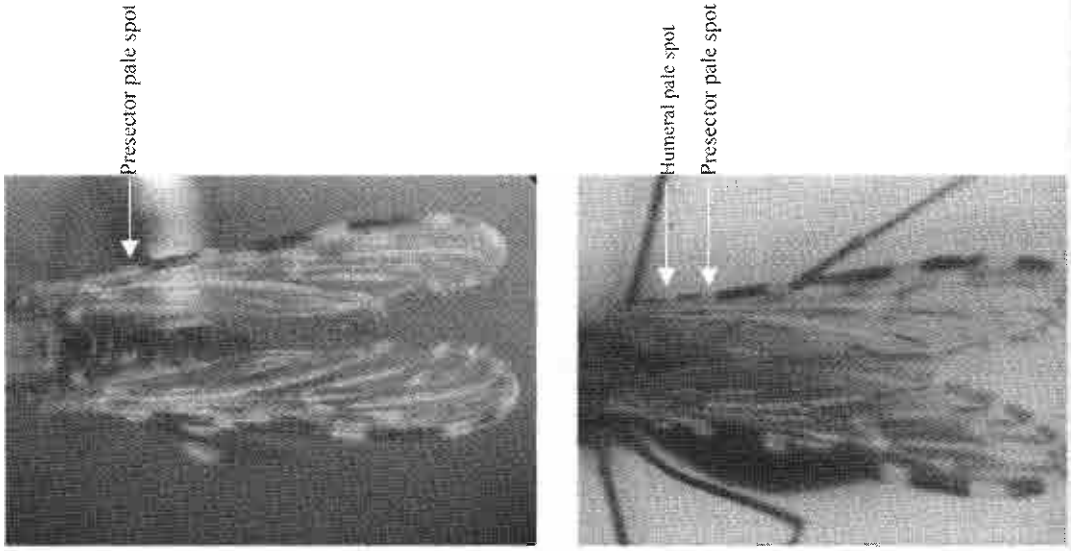

PSP phenotype, *An. minimus* A HP phenotype, *An. minimus* C

Fig. 1. Different spots on the costa of *Anopheles* (Harrison 1980) and pictures of the 2 phenotypes (photos taken by S. S.).

the HP spot on samples from Hoa Binh Province and could not correlate the characters with the sibling species. In southern China, Chen et al. (2002) used the single-strand conformation polymorphism-polymerase chain reaction (SSCP-PCR) assay (Sharpe et al. 1999) to check the morphological identification of *An. minimus* s.l. populations from wild and colony materials. He concluded that this morphological marker was not reliable to distinguish species A from C.

To achieve this study, we considered the previous analyses of Green et al. (1990), Van Bortel et al. (1999), and Chen et al. (2002) together along with a large sample of wild *An. minimus* s.l. populations from 2 sites in Thailand.

MATERIALS AND METHODS

Study areas

The study was conducted in 2 villages each in Kanchanaburi Province, western Thailand, and in Tak Province, northern Thailand. Ban Pu Toei village in Sai Yok district, Kanchanaburi Province, is located in a mountainous area surrounded by forest (14°17N, 99°11E). A 2-m-wide slow-running stream with native vegetation along its margin is the main larval habitat of *An. minimus* s.l. (Sucharit et al. 1988, Green et al. 1990, Chareonviriyaphap et al. 2003). The second site, Ban Nam Dip village in Mae Sot district, Tak Province, is surrounded by a rice field on the east and by a forest on the west

(16°41N, 98°41E). There is a 3-m-wide running stream bordered by a variety of plants all along its margins.

Mosquito collections

In total, 6 populations from different collection methods were obtained. Five populations were from Kanchanaburi Province, namely, TKM, TKi04, TKo04, TKc04, and TKo03; and 1 population was from Tak Province (TT) (Table 1).

TKM population was collected as larvae and pupae in September 2003 by 2 collectors that sampled along the margins of the slow-running stream. After emergence of the adults in the laboratory, only males were kept to assess variability among them.

TKi04 population was obtained from indoor human landing collections made by 2 collectors from 1800 to 0600 h, with an hourly capture period of 45- and 15-min break, during 3 consecutive nights per month from January to August 2004.

TKo04 population was collected by 2 collectors in the same manner as for TKi04, but outdoors from 1800 to 0600 h, 10 m away from the house where indoor landing collection was made from January to August 2004.

TKc04 population was collected from an animal shelter during 3 consecutive nights per month from January to August 2004. Collections were made on 2 cows during 15 min each hour from 1800 to 0600 h.

TKo03 population was collected outdoors by 3 collectors, sitting near the slow-running stream at Ban Pu Toei village in Sai Yok district, Kanchanaburi Province, in August 2003. Collections were made from 1800 to 2200 h during 3 consecutive nights.

TT population is the only population collected at Ban Nam Dip village, Tak Province, in June and August 2003, during 3 consecutive nights each month, by 2 collectors sitting approx. 20 m away from a house.

Morphological identification

All mosquitoes were kept alive after the collections and subjected to species identification the next day in the laboratory (Department of Entomology, Kasetsart University, Thailand) by using the morphological keys of Peyton and Scanlon (1966) and Rattanarithikul and Punthusiri (1994). Specimens belonging to *An. minimus* s.l. were identified as species A if the PSP phenotype was present and as species C if the HP phenotype was present (Fig. 1).

Molecular identification

Specimens of *An. minimus* s.l. were individually DNA extracted according to the procedure of Collins et al. (1987). Molecular identification was done using the allele-specific PCR (AS-PCR) assay of Garros et al. (2004a). To check misidentifications

Table 1. Population characteristics.[1]

Population code	Country, locality	Collection date and methods	Sex	*n*	Molecular identification	Reference
TKM	Thailand, Kanchanaburi Province	September 2003, immature collection	M	37	AS-PCR	Present work
TKi04		January–August 2004, monthly indoor human landing collections	F	50		
TKo04		January–August 2004, monthly outdoor human landing collections	F	102		
TKc04		January–August 2004, monthly cattle collections	F	121		
TKo03		Aug. 2003, outdoor human landing collection	F	63		
TT	Thailand, Tak Province	June and August 2003, outdoor human landing collections	F	27		
Total				400		
TG	Thailand, Kanchanaburi Province	1984 and 1987, human landing, cattle, and immature collections	F	263	Isozyme	Green et al. 1990
VVB	Vietnam, Hoa Binh Province	June–November 1995, indoor and outdoor human landing, cattle, and indoor resting collections	F	911		Van Bortel et al. 1999
CC	China, several localities in southern China	July–September 2000, August–September 2001, human landing, cattle, and immature collections	M	256	SSCP-PCR	Chen et al. 2002
Grand total				1,830		

[1] The population codes are as follows: C, China; T, Thailand; V, Vietnam; K, Kanchanaburi Province; T, Tak Province; i, indoor human landing collection; o, outdoor human landing collection; c, cattle collection. The last 2 numbers correspond to the collection year. For TG, VVB, and CC populations, the last letter(s) is for the first author. Sex: M, male; F, female.

with the other species of the Minimus Group (*Anopheles aconitus, Anopheles pampanai,* or *Anopheles varuna*), specific primers of all 5 species were multiplexed.

Data analysis

To test the reliability of the presence/absence of the HP spot for a clear identification of each species of the Minimus Complex, we used biomedical tests (Altman 1991), which allowed us to evaluate the diagnostic power of the morphological characters. Several values provide insight into the reliability of the test. Sensitivity and specificity were calculated by comparing the observed test outcome with the outcome of the gold standard, i.e., the molecular identification. Another way to characterize a diagnostic test was to calculate the proportion of correctly classified individuals as an index of validity (Iv). The Iv is the probability of agreement between the molecular and the morphological identifications. The positive predictive value (PPV) provides the probability of having an *An. minimus* C specimen if the HP phenotype is present. There is a corresponding negative predictive value (NPV) predicting the probability of rightly identifying *An. minimus* A if the PSP phenotype is present.

RESULTS

In total, 400 mosquitoes were DNA extracted and identified with both morphological and molecular methods (Table 1). Only 1 specimen was morphologically determined as *An. minimus* A, whereas it was molecularly identified as *An. varuna*; therefore, it was deleted from the analysis. The data sets of 3 publications were used as references (Green et al. 1990, Van Bortel et al. 1999, Chen et al. 2002), representing an additional sample of 1,430 *An. minimus* s.l., for a total of 1,830 specimens. All the indexes were calculated and are presented in Table 2.

It is noteworthy that all the specimens (n = 37) of the male population (TKM) from Kanchanaburi Province were misidentified, leading to a null Iv. They were all initially identified as species A but were found as species C with the molecular assay. For the other populations, the index of validity ranged from 0.543 (CC population) to 0.961 (TKo04 population), showing a high probability of agreement (Iv > 0.9) between the molecular and morphological identification for the TKi04 and TKo04 populations. The PPV was maximum (PPV = 1) for the 3 populations TKi04, TKo04, and TKc04, indicating that all the *An. minimus* C of these populations had the HP phenotype. The PPV ranged from 0.690 (CC population) to 0.976 (TKo03 population) for the 5 remaining populations. The NPV, indicating the correct identifications of *An. minimus* A when the PSP phenotype is present, fluctuated between 0.283 (TG population) and 0.667 (VVB population), with a null value for

Table 2. Identification results and indexes.[1]

Population code	Morphological identification	Molecular identification		Specificity	Sensitivity	Iv	PPV	NPV
		Species C	Species A					
TKM	C	0	0	xx	0	0	xx	xx
	A	37	0					
TKi04	C	43	0	1	0.915	0.920	1	0.429
	A	4	3					
TKo04	C	92	0	1	0.958	0.961	1	0.600
	A	4	6					
TKc04	C	89	0	1	0.840	0.860	1	0.469
	A	17	15					
TKo03	C	40	1	0.875	0.727	0.746	0.976	0.318
	A	15	7					
TT	C	23	1	0	0.885	0.852	0.958	0
	A	3	0					
TG	C	131	5	0.878	0.590	0.635	0.963	0.283
	A	91	36					
VVB	C	26	6	0.990	0.082	0.672	0.813	0.667
	A	293	586					
CC	C	20	9	0.930	0.156	0.543	0.690	0.524
	A	108	119					
					Mean	0.774 ± 0.148	0.880 ± 0.125	0.470 ± 0.140

the TT population. This extreme value was most likely due to the low number of identified specimens ($n = 27$). The mean probability of having a correct identification of *An. minimus* A based on the PSP phenotype (mean NPV = 0.47) is significantly lower (Table 2) than the mean probability of carrying a correct identification for *An. minimus* C based on the presence of the HP phenotype (mean PPV = 0.88) ($P < 0.01$).

The PPV was not significantly different when considering the 4 populations collected in the Kanchanaburi Province in 2003 and 2004 (TKo03, TKi04, TKo04, and TKc04) (Table 3). No statistical difference was noted between the PPVs of the 3 collection methods used in Kanchanaburi Province. Therefore, the HP phenotype is not linked to the trophic behavior. The TG population, collected in 1984 and 1987 (Green et al. 1990), was not different from the 5 populations collected in Thailand (the 4 latter populations and TT). This finding may indicate that there are little temporal variations in the HP phenotype in this region. However, the PPV of the TT population (Tak Province) was significantly different from the PPVs of TKo04 and TKo03 populations (Kanchanaburi Province), all collected by outdoor human landing. Moreover, the PPV of the Vietnam (VVB) and China (CC) populations were significantly different from all the other populations, except VVB with TT and CC. These differences may represent spatial variations of the phenotypes.

The NPV was not significantly different between the 3 collecting methods in Kanchanaburi Province (TKi04, TKo04, and TKc04) (Table 3). As well as the HP phenotype, the PSP phenotype does not seem to be linked to the trophic behavior. Significant differences between the NPV of the TG population (collected in 1984 and 1987) and TKi04, TKo04, and TKc04 populations (collected in 2004); and TKo03 (collected in 2003) and TKo04 revealed temporal variations of the PSP phenotype but not between the TG and TKo03. Large spatial variations of the PSP phenotype were noted between the VVB and CC populations and between these 2 populations and the Thai populations.

Table 3. Significance test for the positive predictive (PPV, top) and the negative predictive (NPV, bottom) values.[1]

	TKM	TKi04	TKo04	TKc04	TKo03	TT	TG	VVB	CC
TKM		x	x	x	x	x	x	x	x
TKi04	x		ns	ns	ns	ns	ns	$P < 0.05$[2]	$P < 0.05$
TKo04	x	ns		ns	ns	$P < 0.05$	ns	$P < 0.05$	$P < 0.05$
TKc04	x	ns	ns		ns	ns	ns	$P < 0.05$	$P < 0.05$
TKo03	x	ns	$P < 0.05$	ns		$P < 0.05$	ns	$P < 0.05$	$P < 0.05$
TT	x	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$		ns	ns	$P < 0.05$
TG	x	$P < 0.05$	$P < 0.05$	$P < 0.05$	ns	$P < 0.05$		$P < 0.05$	$P < 0.05$
VVB	x	$P < 0.05$	ns	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$		ns
CC	x	ns	ns	ns	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	

[1] Population codes are as explained in footnote to Table 1.
[2] $P < 0.05$, differ significantly; ns, not significantly different by Kruskall–Wallis test; x, not applicable.

DISCUSSION

Precise identification of anopheline mosquitoes is essential for a better understanding of their potential role in malaria transmission as well as for improving the effectiveness of vector control strategies. Molecular identification assays are really useful tools because they allow rapid and easy identification of numerous mosquitoes in one-shot PCR reaction. However, molecular laboratories are not always available, and chemicals and consumables represent an important budget, especially when a large number of specimens need to be identified. Therefore, the presence of a diagnostic morpholog-

ical character is very important and useful for rapid identification in the field.

Our 2 collecting sites of *An. minimus* s.l. in Thailand were in sympatric areas of both species, in which species C predominated. It will be interesting to add to the data set populations where species C is rare or absent.

Excluding the TKM population, 86.8% in total of the *An. minimus* s.l. were correctly identified, with a high probability (0.880) of identifying *An. minimus* C correctly but a low probability (0.470) of identifying *An. minimus* A correctly, based on the 2 PSP and HP phenotypes. Green et al. (1990) obtained 63% correct identifications and Van Bortel et al. (1999) a higher percentage (67%). Therefore, even if the HP phenotype seems to be present in *An. minimus* C with high reliability, this phenotype also may be present in *An. minimus* A, with high spatial variations. Based on these results, and in agreement with Chen et al. (2002), we suggest that the PSP phenotype should not be used to identify the species of the Minimus Complex. Moreover, the phenotype HP is also present in *An. aconitus, Anopheles jeyporiensis,* and *An. pampanai* (Harrison 1980), species of the Minimus Group (Harbach 1994, 2004). Because the 5 species are very similar, especially at the adult stage, morphological identification based on this polymorphic character will lead to misidentifications. Unpublished data on a large morphological screening (Harbach and Manh, personal communication) revealed that no morphological characters are available to distinguish *An. minimus* A from species C.

Natural populations live under different climates, raising the problem of the influence of temperature on phenotypes. This link has already been revealed in other natural populations of dipterans (Katz and Foley 1993, Dombeck and Jaenike 2004) and anopheline species (Le Sueur and Sharp 1991, Le Sueur et al. 1992). Altitudinal or latitudinal clines were demonstrated (Karan et al. 1998, 2000; Gibert et al. 2004). Our results exhibited spatial variation within the Thai populations from Kanchanaburi and Tak Provinces and among the populations of the 3 countries (China, Thailand, and Vietnam), which may reflect different ecological and climatic conditions. The divergence is less marked when closed populations are compared, especially for the HP phenotype. The relative high homogeneity of the populations in Kanchanaburi over the 2 years studied could be explained by a relative climatic stability of the environment in which the site is localized. The function and role of the wing spots are unknown. They might play a role in 1) camouflage, 2) communication and recognition between and within species, or 3) protection against solar radiation. Regardless, the morphological variation suggests response to local conditions.

Moreover, several studies have suggested the influence of seasonality on the color patterns observed on *Anopheles* adults. Davis (1928), working on the *Nyssorhynchus* species in South America, concluded that melanism was correlated to seasons, with darker patterns being dominant during colder months. This relationship also was confirmed by Le Sueur and Sharp (1991) on *Anopheles merus.* In the Afrotropical region, Leeson (1930), Gillies (1963), and Service (1964) also concluded that the dark or pale scaling on wing veins may be governed by climatic changes linked to the seasons. In India, climatic variants of *Anopheles fluviatilis* s.l. also were found during the cool season (Rahman et al. 1960). In northern Thailand, Harrison (1980) observed that adults of *An. aconitus* and *An. minimus* s.l. were darker during the cool season (November–January). More specifically for *An. minimus* s.l., a color cline over Southeast Asia was stated, with a darkening trend for the northern latitudes. This finding also was supported by Liu et al. (1959). Harrison (1980) proposed that the temperature of the breeding sites may influence the morphological traits. Recently, Van Bortel et al. (1999) noted a change in the relative importance of the different morphotypes in each of the *An. minimus* species during the study period (from wet to cool dry season). In Thailand, 3 periods are recognized: from late November to early February with cool and dry weather, from February to May with hot and dry weather, and from June to November with the rainy season. Unfortunately, it was not possible to test this hypothesis either with our samples or with the samples of Green et al. (1990) (collected in January 1984 and May–June 1987) because the data were pooled.

Green et al. (1990) and Van Bortel et al. (1999) checked the morphological identifications with an isozyme assay (Green et al. 1990), whereas Chen et al. (2002) used the SSCP-PCR (Sharpe et al. 1999). These techniques presented potential reading errors, which also could explain significant differences among the populations.

In agreement with Chen et al. (2002), we concluded that the wing spot patterns present variation that makes them unreliable and unsuitable as diagnostic characters to clearly identify both species A and C of the Minimus Complex. For such cryptic species, where no reliable morphological character is diagnostic, molecular identification remains mandatory and a more appropriate and robust method to obtain an unambiguous differentiation.

ACKNOWLEDGMENTS

This investigation received financial support from the International Master Program (Kasetsart University, French Embassy, Thailand and Institute of Research for Development), the Thailand Research Funds, and the Kasetsart University Research and Development Institute.

REFERENCES CITED

Altman DG. 1991. *Some common problems in medical research. Practical statistics for medical research.* London: Chapman & Hall.

Chareonviriyaphap T, Prabaripai A, Bangs MJ, Aum-Aung B. 2003. Seasonal abundance and blood feeding activity of *Anopheles minimus* Theobald (Diptera: Culicidae) in Thailand. *J Med Entomol* 40:876–881.

Chen B, Harbach RE, Butlin RK. 2002. Molecular and morphological studies on the *Anopheles minimus* group of mosquitoes in southern China: taxonomic review, distribution and malaria vector status. *Med Vet Entomol* 16:253–265.

Collins FH, Mendez MA, Rasmussen MO, Mehaffey PC, Besansky NJ, Finnerty V. 1987. A ribosomal RNA gene probe differentiates member species of the *Anopheles gambiae* complex. *Am J Trop Med Hyg* 37:37–41.

Davis NC. 1928. A consideration of variability in the *Nyssorhynchus* group of the genus *Anopheles*. *Am J Hyg* 8:539–563.

Dombeck I, Jaenike J. 2004. Ecological genetics of abdominal pigmentation in *Drosophila falleni*: a pleiotropic link to nematode parasitism. *Int J Org Evol* 58:587–596.

Garros C, Koekemoer LL, Coetzee M, Coosemans M, Manguin S. 2004a. A single multiplex assay to identify major malaria vectors within the African *Anopheles funestus* and the Oriental *An. minimus* groups. *Am J Trop Med Hyg* 70:583–590.

Garros C, Koekemoer LL, Kamau L, Awolola TS, Van Bortel W, Coetzee M, Coosemans M, Manguin S. 2004b. Restriction fragment length polymorphism method for the identification of major African and Asian malaria vectors within the *Anopheles funestus* and *An. minimus* groups. *Am J Trop Med Hyg* 70:260–265.

Gibert P, Capy P, Imasheva A, Moreteau B, Morin JP, Petavy G, David JR. 2004. Comparative analysis of morphological traits among *Drosophila melanogaster* and *D. simulans*: genetic variability, clines and phenotypic plasticity. *Genetica* 120:165–179.

Gillies MT. 1963. A note on the identification of *Anopheles rivulorum* Leeson (Diptera: Culicidae). *Proc R Entomol Soc Lond B* 32:86–88.

Green CA, Gass RF, Munstermann LE, Baimai V. 1990. Population-genetic evidence for two species in *Anopheles minimus* in Thailand. *Med Vet Entomol* 4:25–34.

Harbach RE. 1994. Review of the internal classification of the genus *Anopheles* (Diptera: Culicidae): the foundation for comparative systematics and phylogenetic research. *Bull Entomol Res* 84:331–342.

Harbach RE. 2004. The classification of genus *Anopheles* (Diptera: Culicidae): a working hypothesis of phylogenetic relationships. *Bull Entomol Res* 94:537–553.

Harrison BA. 1980. The *Myzomyia* Series of *Anopheles* (*Cellia*) in Thailand, with emphasis on intra-interspecific variations (Diptera: Culicidae). Medical entomology studies -XIII. *Contr Am Entomol Inst* 17:1–195.

Karan D, Dubey S, Moreteau B, Parkash R, David JR. 2000. Geographical clines for quantitative traits in natural populations of a tropical drosophilid: *Zaprionus indianus*. *Genetica* 108:91–100.

Karan D, Munjal AK, Gibert P, Moreteau B, Parkash R, David JR. 1998. Latitudinal clines for morphometrical traits in *Drosophila kikkawai*: a study of natural populations from the Indian subcontinent. *Genet Res* 71:31–38.

Katz AJ, Foley TA. 1993. Effect of temperature on frequencies of spots in *Drosophila* wing-spot assay. *Environ Mol Mutagen* 22:54–58.

Le Sueur D, Sharp BL. 1991. Temperature-dependent variation in the head capsule width and wing length of *Anopheles merus* Dönitz: implications for anopheline taxonomy. *Med Vet Entomol* 5:55–62.

Le Sueur D, Sharp BL, Appleton CC. 1992. Dark-scaled areas on adult *Anopheles* mosquitoes are selectively affected by temperature-related size variation. *Med Vet Entomol* 6:396–398.

Leeson HS. 1930. Variations in the wing ornamentation of *Anopheles funestus* Giles. *Bull Entomol Res* 21:421–425.

Liu LC, Fang CC, Hu MH. 1959. Study on the forms of *Anopheles minimus* Theobald, 1901. *Acta Entomol Sin* 9:154–160.

Peyton EL, Scanlon JE. 1966. *Illustrated key to the female* Anopheles *mosquitoes of Thailand.* Bangkok, Thailand: U.S. Army Med Component South East Asia Treaty Organization.

Phuc HK, Ball AJ, Son L, Hanh NV, Tu ND, Lien NG, Verardi A, Townson H. 2003. Multiplex PCR assay for malaria vector *Anopheles minimus* and four related species in the Myzomyia Series from Southeast Asia. *Med Vet Entomol* 17:423–428.

Rahman J, Singh MV, Singh NN. 1960. A note on the study of the morphology, prevalence, and host preference of an ecocline of *An. fluviatilis* in Nainital Tarai (UP). *Bull Nat Soc Indian Malariol* 8:137–142.

Rattanarithikul R, Punthusiri P. 1994. Illustrated keys to the medically important mosquitoes of Thailand. *Southeast Asian J Trop Med Public Health* 25:1–66.

Rwegoshora RT, Sharpe RG, Baisley KJ, Kittayapong P. 2002. Biting behavior and seasonal variation in the abundance of *Anopheles minimus* species A and C in Thailand. *Southeast Asian J Trop Med Public Health* 33:694–701.

Service MW. 1964. An analysis of individual and seasonal variations occurring in the wing ornamentation of *Anopheles* (*Cellia*) *funestus* Giles (Diptera: Culicidae). *Proc R Entomol Soc Lond B* 33:139–143.

Sharpe RG, Hims MM, Harbach RE, Butlin RK. 1999. PCR-based methods for identification of species of the *Anopheles minimus* group: allele-specific amplification and single-strand conformation polymorphism. *Med Vet Entomol* 13:265–273.

Somboon P, Tongwat D, Choochote W, Walton C, Takagi M. 2005. Crossing experiments of *Anopheles minimus* species C and putative species E. *J Am Mosq Control Assoc* 21:5–9.

Somboon P, Walton C, Sharpe RG, Higa Y, Tuno N, Tsuda Y, Takagi M. 2001. Evidence for a new sibling species of *Anopheles minimus* from the Ryukyu Archipelago, Japan. *J Am Mosq Control Assoc* 17:98–113.

Sucharit S, Komalamisra N, Leemingsawat S, Apiwathnasorn C, Thongrungkiat S. 1988. Population genetic studies on the *Anopheles minimus* complex in Thailand. *Southeast Asian J Trop Med Public Health* 19:717–723.

Sucharit S, Surathinh K, Chaisri U, Thongrungkiat S, Samang Y. 1995. New evidence for the differed characters of *Anopheles minimus* species complex. *Mosq Borne Dis Bull* 12:1–6.

Van Bortel W, Trung HD, Manh ND, Roelants P, Verle P, Coosemans M. 1999. Identification of two species within the *Anopheles minimus* complex in northern Vietnam and their behavioural divergences. *Trop Med Int Health* 4:257–265.

Van Bortel W, Trung HD, Roelants P, Harbach RE, Backeljau T, Coosemans M. 2000. Molecular identification of *Anopheles minimus s.l.* beyond distinguishing the members of the species complex. *Insect Mol Biol* 9: 335–340.

Influence of nutritional and physiological status on behavioral responses of *Aedes aegypti* (Diptera: Culicidae) to deltamethrin and cypermethrin

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Received 12 September 2005; Accepted 13 November 2005

ABSTRACT: Excito-repellency responses of *Aedes aegypti* (L.) exposed to deltamethrin and cypermethrin were assessed using an excito-repellency test system. Contact irritancy and non-contact repellency assays compared non-bloodfed (unfed) parous (post-gravid), nulliparous, early blood-fed, late blood-fed, sugar-fed, and unmated female mosquitoes for behavioral responses based on nutritional and physiological conditions at the time of testing. Rates of escape during contact exposure with either compound were most pronounced in parous mosquitoes, followed by unmated mosquitoes, when compared to other conditional states. Significantly higher numbers of parous females also escaped from control chambers compared to other cohorts ($P < 0.05$). Irritability of blood- and sugar-fed mosquitoes was noticeably suppressed. We conclude that nutritional and physiological conditions (including age) of mosquitoes at the time of testing can significantly influence behavioral responses (excito-repellency) to insecticides. The findings indicate that whether due to chronological age, nutrition, physiological state, or innate (circadian) activity patterns, careful consideration must be given to the selection of appropriate conditioned mosquitoes for testing. ***Journal of Vector Ecology* 31 (1): 89-101. 2006**

Keyword Index: *Aedes aegypti*, excito-repellency, behavior, deltamethrin, cypermethrin.

INTRODUCTION

Dengue hemorrhagic fever is one of the most serious viral illnesses of humans, occurring worldwide in many tropical/subtropical regions and placing 50-100 million people at risk of serious infection each year (Gubler 1997). The primary vector is the day-biting mosquito, *Aedes (Stegomyia) aegypti* (L.), an eusynanthropic mosquito that typically breeds in and around human habitation and seeks blood primarily from humans. Prevention and control of dengue transmission still relies heavily on control of the vector's preferred larval habitats. Elimination or resource availability management ("source reduction") of larval habitats has been the most common approach to vector control but remains an expensive endeavor that is difficult to sustain without large budgets, commitment, and active community participation (Gubler 1997). Adult vector control using insecticides applied as transitory space sprays, usually in direct response to dengue outbreaks, has been commonly used in and around homes for decades and is believed to be an important contributor of insecticide resistance in house-haunting mosquitoes like *Ae. aegypti*.

Although some populations of *Ae. aegypti* in Thailand have been found to be physiologically resistant to several synthetic compounds (Chareonviriyaphap et al. 1999, Somboon et al. 2003), the true impact of resistance on vector control and disease transmission has not been adequately clarified (WHO 1992, Kongmee et al. 2004). Apart from the toxicological action, many synthetic pyrethroids have been shown to have irritant or repellent properties to insects (Threlkeld 1985). Most reports have focused on the excito-repellency properties of DDT and synthetic pyrethroids against *Anopheles* species (Coosemans and Sales 1977, Pell et al. 1989, Roberts et al. 2000, Chareonviriyaphap et al. 1997, 2001). Relatively little interest has been paid to behavioral responses of *Ae. aegypti* exposed to insecticides (Kennedy 1947, Lal et al. 1965, Moore 1977) and only one study has described the two principal types of behavioral responses, irritancy and repellency (Kongmee et al. 2004) in this species using a standardized excito-repellency test chamber and analysis (Roberts et al. 1997, Chareonviriyaphap et al. 2002). The influence of nutritional and physiological conditions on behavioral responses of *Ae. aegypti* during exposure to insecticides has not been investigated (Sungvornyothin et al. 2001), thus forming the objective of this study. The excito-repellency response of *Ae. aegypti* to deltamethrin and cypermethrin was determined by directly comparing the numbers of female mosquitoes escaping from test chambers following exposure to synthetic pyrethroids under controlled

laboratory conditions.

MATERIALS AND METHODS

Mosquito populations

A colony of *Chiangmai* population was established from larvae and pupae from Pang Mai Daeng Village, Mae Taeng District, Chiangmai Province (14° 15' N, 99° 17' E) in August 2004. The colony was kept at the Department of Entomology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand. The F1-F3 generations were used for excito-repellency (ER) tests.

A colony of *Kanchanaburi* population was established from larvae and pupae collected from Pu Teuy Village, Sai Yok District, Kanchanaburi Province (14° 17' N, 99° 11'E) in September 2004, and kept at the Department of Entomology, Kasetsart University. The F1-F3 generations were used for ER tests.

Mosquito rearing and conditioning

Colonization of *Aedes aegypti* from field collections followed established methods (Kongmee et al. 2004) with only minor modifications to meet testing requirements. All life stages were maintained under insectary conditions before, during, and following testing. Larval and adult insects were kept under a 12:12 light:dark photoperiod regime. Adults were provided cotton pads soaked with 10% sucrose solution from the first day of emergence. Adults were held in 30 cm^3 screen-meshed cages. Depending on required experimental conditions, female mosquitoes were permitted to have a blood meal (live hamster) on the third or fourth day post-emergence. Two days post-blood feeding, oviposition dishes were placed in the cage for gravid females to deposit eggs.

Six different nutritional and physiological conditions of female mosquitoes of varying ages were used in this study. Cohorts included: *Parous* (mated) females blood-fed on live hamsters on day 3, held for 3 days until oviposition, and then held for an additional 3 days with only water provided before testing (between 8 and 9-days-old). *Nulliparous* (mated) mosquitoes were denied blood and sugar, provided water only, and were 2 to 3-days-old at the time of testing. *Early blood-fed* and *Late blood-fed* mosquitoes were 4-days and 6-days-old, respectively. Only fully blood-fed mosquitoes were used. *Sugar-fed* (10% sucrose only) mosquitoes were 3 to 4-days-old at testing. *Unmated* females were obtained by placing individual pupae in separate containers until emergence, after which female adults were segregated into all-female cages until testing. Infertile females were provided water only and were 3 to 4 days of age at the time of testing. All six conditioned cohorts were kept separate before, during, and after testing.

Insecticides

Two synthetic pyrethroid insecticides were used in excito-repellency assays: Deltamethrin [(S)-alpha-cyano-3-phenoxybenzyl (1R,3R)-3-(2,2-dibromovinyl)-2,2-dimethyl cyclopropane carboxylate] (85% purity) provided by Bayer, Thailand, and cypermethrin [RS-alpha-cyano-3-phenoxybenzyl (1RS,3RS;1RS,3SR)-3-(2,2-dichlorovinyl)-2,2- dimethylcyclopropanecarboxylate] (87% purity) supplied by Ladda Company, Thailand.

Insecticide papers

Test papers (27.5 x 35.5 cm^2) were chemically impregnated using World Health Organization (WHO 1998) recommended diagnostic concentrations of deltamethrin (0.05%) and cypermethrin (0.5%). For behavioral avoidance assays, operational field doses of deltamethrin (0.02g/m^2) and cypermethrin (0.5 g/m^2) for *Ae. aegypti* were used (WHO 1998). Treated papers were prepared by the Center for Vaccine Development, Institute of Science and Technology for Research and Development, Mahidol University, Salaya District, Nakhonpathom Province, Thailand, according to specification (WHO 1996). All insecticide papers were treated at the rate of 2.75 ml insecticide solution per 180 cm^2 surface area. Control papers were treated with acetone (solvent) plus silicone oil.

Insecticide susceptibility test

The susceptibility of each population to each insecticide at the concentrations indicated were assessed by exposing unfed, 3-5-d-old female mosquitoes to insecticide-treated test papers following standard testing procedures and exposure times (WHO 1998). For each test, five test cylinders (two controls and three treatments) were used. Control cylinders contained filter paper impregnated with solvent and carrier; treatments contained paper impregnated with the diagnostic concentration of insecticide and solvent. Twenty-five mosquitoes were introduced into each cylinder for 1 h. Mosquitoes were then transferred to holding containers, and a 10% sucrose solution was provided. Mortalities were recorded at 24 h. Each test was replicated four times.

Behavioral tests

The test system used in this study is described in detail by Chareonviriyaphap et al. (2002). A complete test required four separate chambers; two pairs of treatment and control chambers, respectively. All tests were conducted between 0800 to 1630 h under natural conditions of temperature and relative humidity (range 27-32° C and 50-75% relative humidity). Two different strains of six different conditioned cohorts each were used in this study: parous, nulliparous, late blood-fed, early blood-fed, sugar-fed, and unmated mosquitoes of varying ages. All trial sets were replicated at least three times.

The general test procedures described by Kongmee et al. (2004) were followed with only minor modifications to meet experimental protocol. Twenty-five mosquitoes were carefully introduced into each of four chambers using a mechanical aspirator. A receiving cage (20 x 27 x 24 cm paper box) was connected to the exit portal for collecting mosquitoes that escaped. Mosquitoes were allowed a 3-min resting period to adjust to the darkened inside of the test chamber before the escape funnel was opened and the observation period begun. Numbers of mosquitoes escaping from the chambers into the receiving cage were recorded at 1-min intervals for 30 min. The number of dead or moribund (knockdown) specimens,

Table 1. Mean susceptibility of two strains of *Ae. aegypti* at diagnostic concentrations of deltamethrin and cypermethrin.

Strain of *Ae. aegypti*	Deltamethrin		Cypermethrin	
	No. Tested	% Mortality	No. Tested	% Mortality
Chiangmai	300	180 (60)	300	120 (40)
Kanchanaburi	300	300 (100)	300	45 (15)

either remaining inside the chamber or those escaping to the receiving cage, were recorded separately for treated and control chambers. All live mosquitoes in or escaped were collected, provided sucrose solution, and held in separate containers to record mortality over a 24-h post-exposure period.

Data analysis

Kaplan-Meier survival analysis was used to examine the excito-repellency data collected at 1-min intervals (Roberts et al. 1997). Survival analysis was used to derive escape time (ET) percentage estimates (in min) for the different insecticides and conditions of test mosquitoes. A log-rank method was used to compare the patterns of escape behavior between the two mosquito populations and various nutritional and physiological conditions (Mantel and Haenzel 1959). Statistical significance was set at 0.05 level of probability.

RESULTS

The percent mortality of adult *Ae. aegypti* at the single diagnostic dosage is given in Table 1. Both populations showed partial resistance to either deltamethrin or cypermethrin, or both. *Chiangmai* demonstrated 60% and 40% mortality following standard exposure times to deltamethrin and cypermethrin, respectively. Deltamethrin produced 100% mortality in *Kanchanaburi* (complete susceptibility); whereas cypermethrin resulted in only 15% death.

There were marked escape patterns of *Ae. aegypti* after contact with either of the two chemicals as compared to control and noncontact trials and regardless of physiological or nutritional condition at the time of the test ($P < 0.05$). In all cases, no significant differences in escape patterns were found between non-contact trials and paired controls ($P > 0.05$). Among the controls, significantly high numbers of post-gravid (parous) mosquitoes departed untreated chambers ($P < 0.05$) compared to other conditioned cohorts (e.g., *Kanchanaburi* 55-61% control mosquitoes escaped within 30 min). However, there was significant difference in escape patterns between contact and control trials for parous females ($P<0.05$). In general, mortality of escaped mosquitoes was less with deltamethrin vs. cypermethrin (Tables 2 and 3). Relatively low numbers of mosquitoes escaped in noncontact trials exposed to either compound (data not shown).

Table 2 shows contact escape responses and percent

Table 2. Percent mortality of parous, nulliparous, late blood-fed, early blood-fed, sugar-fed, and unmated *Ae. aegypti* (Kanchanaburi) after contact with deltamethrin and cypermethrin.

Insecticide	Number				% Mortality			
	Treatment		Control		Treatment		Control	
	#	%esc	#	%esc	Esc	Rem	Esc	Rem
Deltamethrin								
Parous	71	65	69	55	9	11	0	0
Nulliparous	100	31	100	8	2	0	0	0
Late blood-fed	100	24	100	1	0	0	0	0
Early blood-fed	100	18	100	5	0	1	0	0
Sugar-fed	100	21	98	0	1	5	0	0
Unmated	100	47	98	2	0	1	0	0
Cypermethrin								
Parous	92	85	99	61	16	3	0	0
Nulliparous	97	43	98	1	2	8	0	0
Late blood-fed	99	23	100	1	5	16	0	0
Early blood-fed	100	13	97	3	2	19	0	0
Sugar-fed	97	24	99	4	1	4	0	0
Unmated	75	45	73	10	3	9	0	0

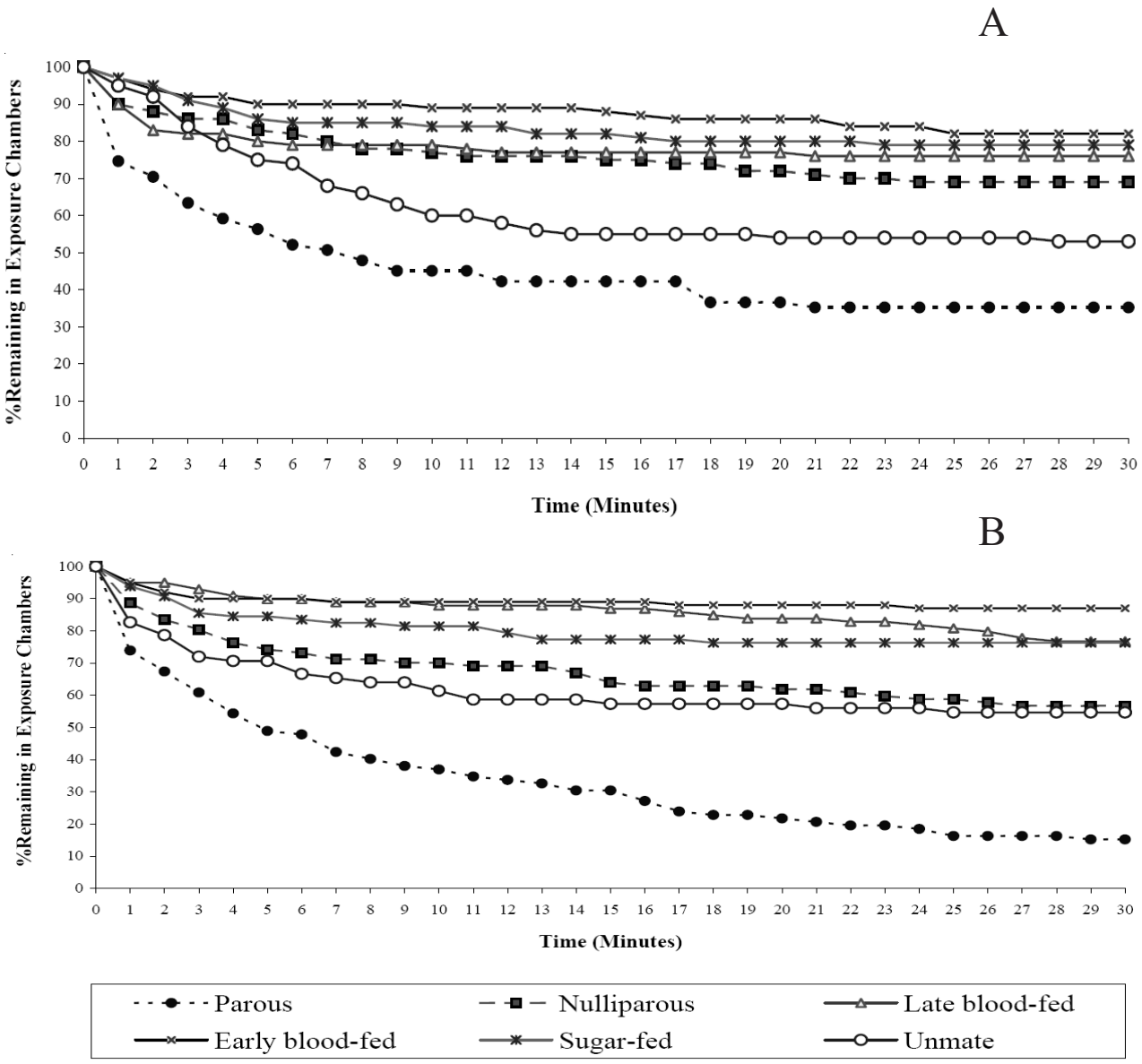

Figure 1. Proportion of parous, nulliparous, late blood-fed, early blood-fed, sugar-fed, and unmated female *Ae. aegypti* (Kanchanaburi) remaining in exposure chambers during contact trials with deltamethrin (A) or cypermethrin (B).

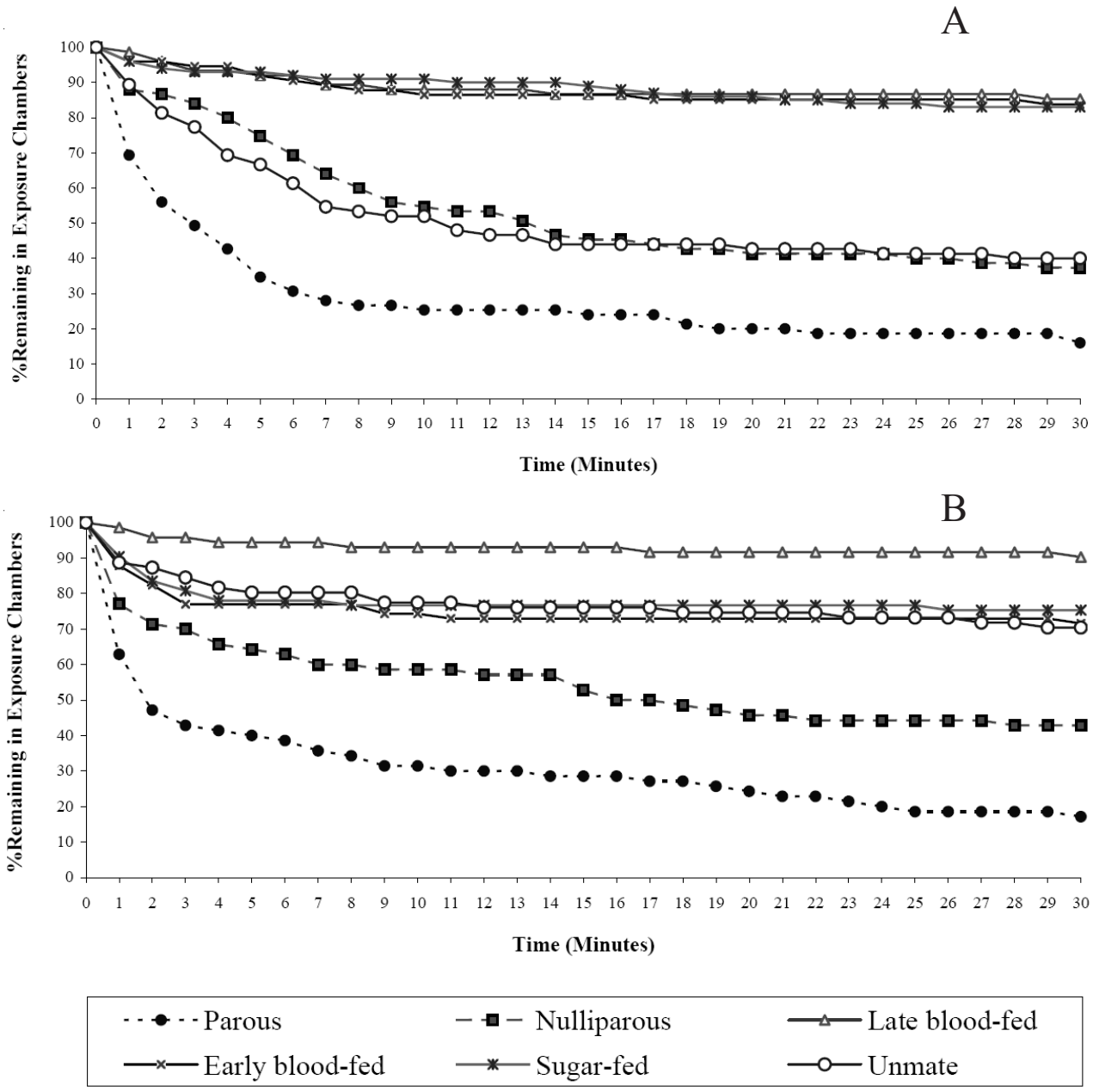

Figure 2. Proportion of parous, nulliparous, late blood-fed, early blood-fed, sugar-fed, and unmated female *Ae. aegypti* (Chiangmai) remaining in exposure chambers during contact trials with deltamethrin (A) and cypermethrin (B).

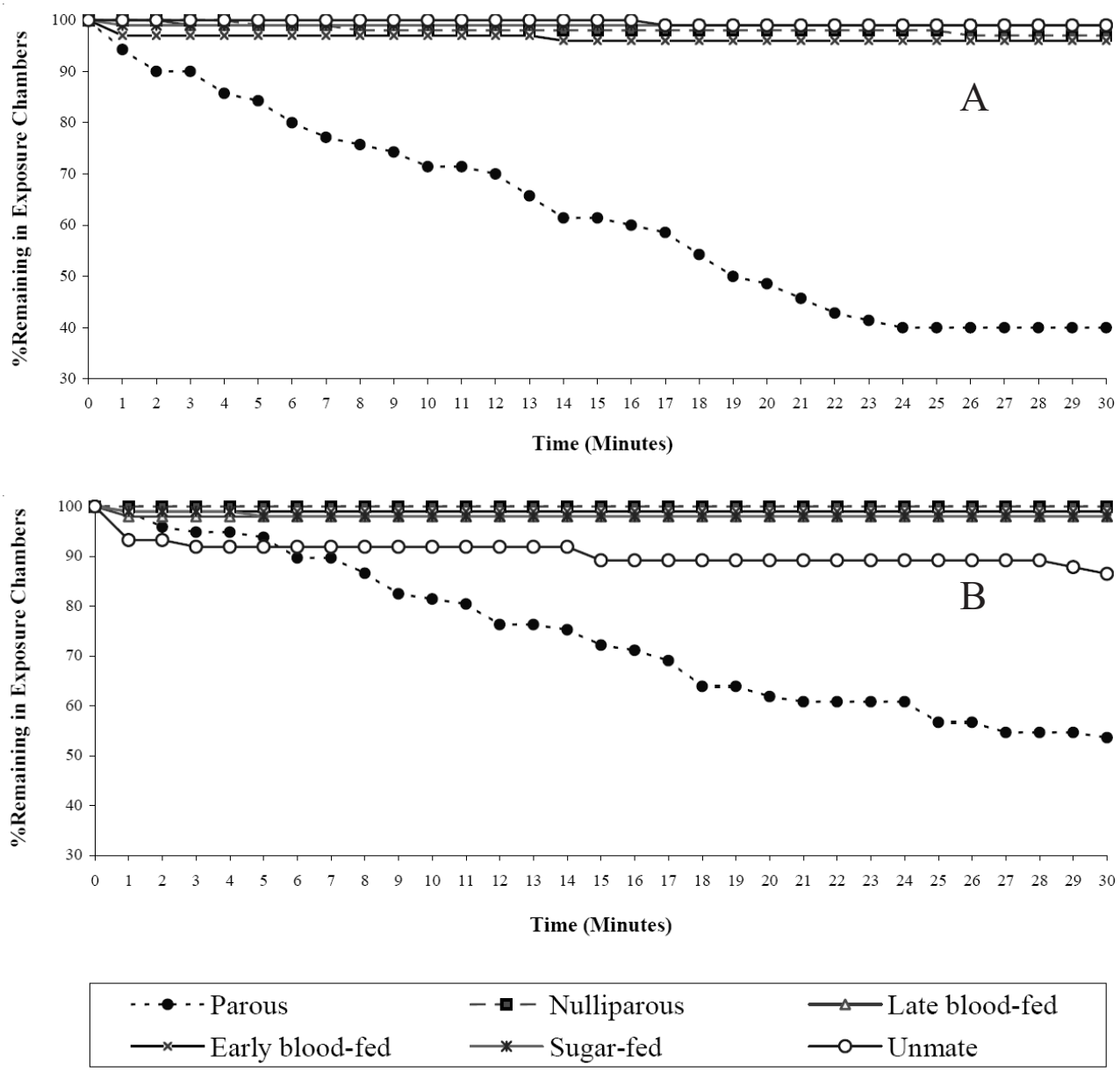

Figure 3. Proportion of parous, nulliparous, late blood-fed, early blood-fed, sugar-fed, and unmated female *Ae. aegypti* (Kanchanaburi) remaining in exposure chambers during non-contact trials with deltamethrin (A) and cypermethrin (B).

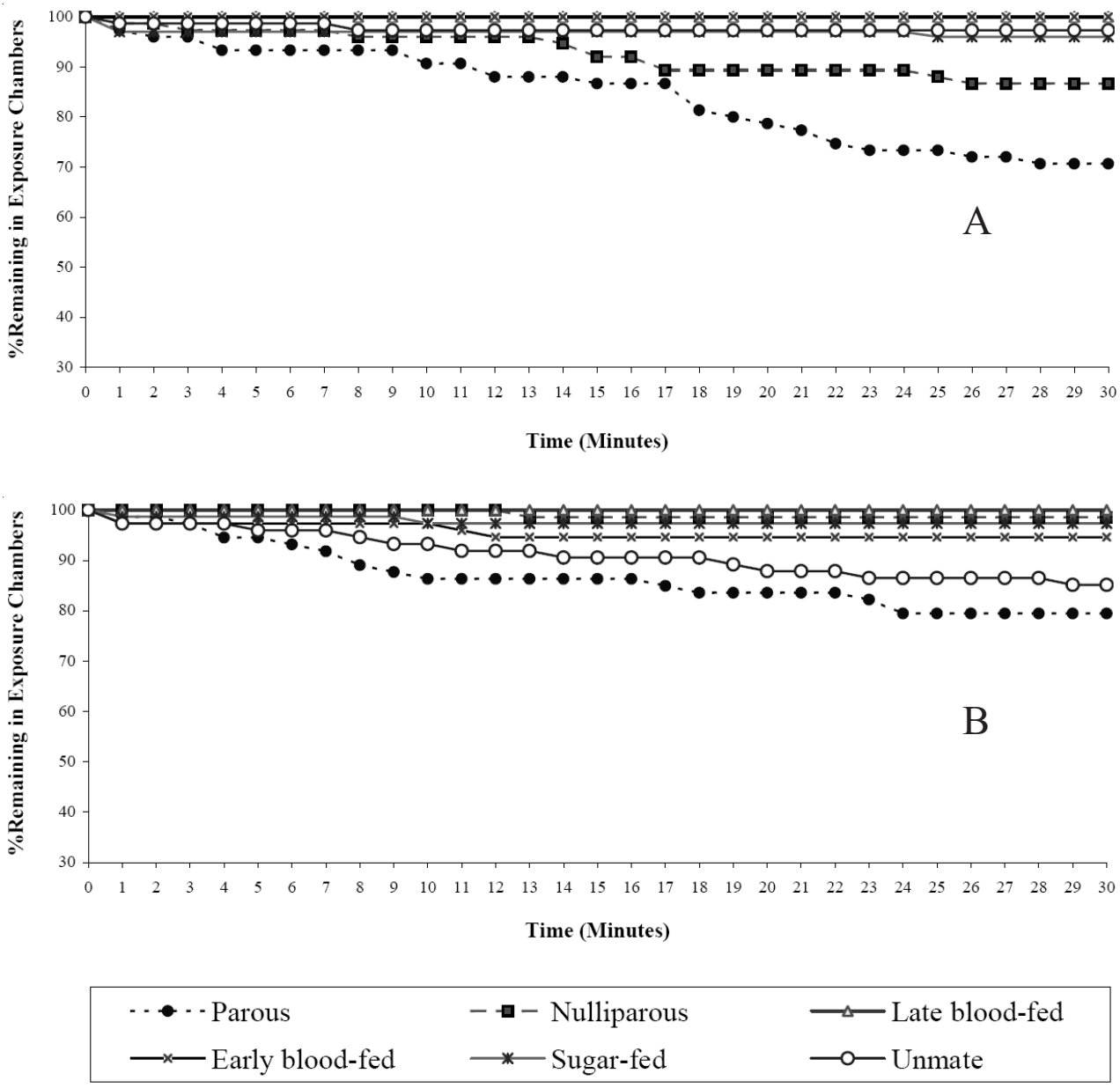

Figure 4. Proportion of parous, nulliparous, late blood-fed, early blood-fed, sugar-fed, and unmated female *Ae. aegypti* (Chiangmai) remaining in exposure chambers in non-contact trials with deltamethrin (A) and cypermethrin (B).

Table 3. Percent mortality of parous, nulliparous, late blood-fed, early blood-fed, sugar-fed, and unmated *Ae. aegypti* (Chiangmai) after contact with deltamethrin and cypermethrin.

	Number				%Mortality			
Insecticide	Treatment		Control		Treatment		Control	
	#	%esc	#	%esc	Esc	Rem	Esc	Rem
Deltamethrin								
Parous	75	84	75	29	1	0	0	0
Nulliparous	75	63	74	22	0	0	0	0
Late bloodfed	75	15	75	0	0	0	0	0
Early bloodfed	74	16	75	0	0	0	0	0
Sugarfed	100	17	100	2	0	6	0	0
Unmated	75	60	75	7	0	1	0	0
Cypermethrin								
Parous	70	83	71	28	30	13	0	0
Nulliparous	70	57	70	39	39	30	1	3
Late bloodfed	72	10	74	0	3	51	0	0
Early bloodfed	74	28	75	3	12	55	0	0
Sugarfed	73	25	74	4	8	43	0	0
Unmated	71	30	73	15	13	58	0	0

mortalities of *Kanchanaburi* test populations under various nutritional and physiological conditions following a 24-h holding period. The highest escape response was observed in parous females (65% deltamethrin, 85% cypermethrin), while the lowest was seen in early bloodfed mosquitoes (18% deltamethrin, 13% cypermethrin). A large percentage (47%) of unmated mosquitoes also escaped from deltamethrin-treated chambers. Percent mortality following 24 h was low for all tests, ranging from 0 to 19%.

Contact escape responses for *Chiangmai* mosquitoes at various nutritional and physiological conditions were similar for both chemicals (Table 3). As with *Kanchanaburi*, the greatest escape response was observed in parous mosquitoes (84% deltamethrin, 83% cypermethrin), whereas the lowest response was observed in the late bloodfed cohorts (15% deltamethrin, 10% cypermethrin). With deltamethrin, 60 and 63% of unmated and nulliparous females, respectively, exited treated chambers. For *Chiangmai*, the percent escape from control chambers ranged from 0 to 39%, the majority being parous mosquitoes. Percent mortalities were higher with cypermethrin contact than deltamethrin, especially among sugarfed (43%), early bloodfed (55%), and late bloodfed (51%).

Multiple paired log-rank comparisons of escape in contact trials were analyzed (data not shown). Significant differences were found for all comparisons of parous females with other cohorts ($P < 0.05$). With deltamethrin, statistical differences were observed with unmated females among all others ($P < 0.05$), except for nulliparous *Chiangmai* females ($P > 0.05$). Significant differences in escape were also seen with unmated females compared to parous and nulliparous mosquitoes exposed to cypermethrin.

Time in minutes for mosquitoes to escape from contact chambers treated with deltamethrin and cypermethrin were estimated (Table 4). Escape time patterns were set at 30% (ET30) and 60% (ET60) of the test population departing the test chamber during the 30-min period. *Chiangmai* ET30 and ET60 values for parous females were rapid, 1 and 4 min for deltamethrin and 1 and 5 min for cypermethrin. The ET30 for nulliparous females was 6 min for deltamethrin and 3 min for cypermethrin, and for unmated females, 4 min for deltamethrin (Table 4). *Kanchanaburi* ET30 and ET60 values were more dramatic than *Chiangmai*, but were similar in pattern; for parous mosquitoes 2 and 17 min for deltamethrin and 2 and 8 min for cypermethrin. The ET30 for nulliparous were 22 min for deltamethrin and 10 min cypermethrin and for unmated females 7 min for deltamethrin and 5 min for cypermethrin. The ET30 and ET60 in late blood-fed, early

Table 4. Time in minutes for 30% (ET30) and 60% (ET60) parous, nulliparous, late bloodfed, early blood-fed, sugar-fed, and unmated *Ae. aegypti* to escape from excito-repellency chambers treated with deltamethrin and cypermethrin during 30 min contact trials.

Population	Condition	Deltamethrin		Cypermethrin	
		ET30	ET60	ET30	ET60
Chiangmai	Parous	1	4	1	5
	Nulliparous	6	-	3	-
	Unmated	4	-	-	-
Kanchanaburi	Parous	2	17	2	8
	Nulliparous	22	-	10	-
	Unmated	7	-	5	-

All other physiological conditions (not indicated) did not leave within 30 min.

blood-fed, and sugar-fed cohorts could not be calculated because the few numbers that escaped precluded ET analysis.

Figures 1 to 4 illustrate the proportions of mosquitoes remaining in the exposure chambers defined by physiological and nutritional conditions. The proportions were used to develop probabilities of escape under contact with deltamethrin or cypermethrin for both *Kanchanaburi* (Figure 1) and *Chiangmai* populations (Figure 2). Contact escape patterns for parous females were consistently and significantly greater than those of other nutritional states across both compounds in both populations ($P<0.05$). However, dramatic escape responses were also observed in parous controls compared to other control cohorts ($P<0.05$). Significant differences were noted between all contact and paired control trails, except for *Kanchanaburi* parous females and paired controls with either compound. Noncontact repellency was less dramatic a response compared to irritancy. In noncontact trials, *Kanchanaburi* escape patterns for parous females were statistically greater than those of other cohorts (Figure 3); whereas, escape response for parous females was less pronounced in *Chiangmai* (Figure 4). As seen in contact experiments, significantly higher numbers of parous mosquitoes departed the control chambers in noncontact trials.

DISCUSSION

The natural tendency for mosquitoes to avoid insecticide-treated surfaces appears to be a general phenomenon, yet behavioral responses of insects exposed to insecticides remain poorly studied and understood. We believe this area of research has been relatively neglected yet remains an important aspect to understanding how vector control methods function and for sound decision-making on pesticide selection (Muirhead-Thomson 1960, de Zulueta 1964, Roberts et al. 2000). Mosquito behavior is of prime epidemiological importance to the extent it either favors or arrests a mosquito feeding on a human, potentially imbibing an infectious blood meal or transmitting a pathogen to a susceptible host (Elliott 1972). Historically, the general notion that behavioral avoidance by mosquito vectors in the presence of insecticides is considered a detriment to effective mosquito vector control programs has not been adequately tested (Davidson 1953, Rawlings and Davidson 1982, Quinones and Suarez 1989, Ree and Loong 1989). Evidence to the contrary has shown that excito-repellency and the interference of normal mosquito behavior caused by toxic residues can actually enhance control efforts by reducing vector-human contact and thus the risk of pathogen transmission (Roberts and Andre 1994, Roberts et al. 2000). Quantitatively, the combined effects of insecticidal repellency and irritancy can potentially exert the more dominant and most persistent actions of a chemical on an insect versus the lethal properties that are more often cited. For example, in Brazil, the excito-repellency action of DDT afforded almost complete protection of humans from *Anopheles darlingi* residing indoors for nearly two months following house spraying (Roberts and Alecrim 1991). Long recognized in importance, the actual amount of study on mosquito behavior has been inadequate in relation to insecticide assessment and the impact of behavioral avoidance on reducing disease transmission (Muirhead-Thomson 1960, Mattingly 1962).

Studies on the excito-repellency properties of insecticides have focused almost exclusively on responses of anopheline mosquitoes (Coosemans and Sales 1977, Pell et al. 1989, Roberts et al. 2000, Chareonviriyaphap et al. 2001, 2004). Comparatively, little has been reported on behavioral responses of *Ae. aegypti* to toxic compounds (Kennedy 1947, Moore 1977), and only recently has an excito-repellency evaluation of *Ae. aegypti* to a pyrethroid (deltamethrin) been documented (Kongmee et al. 2004). The behavioral response of nine different test populations of *Ae. aegypti* exposed to deltamethrin was the first to quantify excito-repellency in this mosquito. As follow-up, the study described herein explored the same responses in two populations *Ae. aegypti* to two pyrethroids under different nutritional and physiological conditions.

Two types of insect behavioral responses to insecticides have been defined, namely irritability and repellency (Davidson 1953, Roberts and Andre 1994, Rutledge et al. 1999). Irritability is described as an insect's response of leaving an insecticide-treated surface following physical contact with the chemical; whereas, repellency refers to a function of a compound to influence an avoidance response from a distance without actual physical contact, thereby diverting insects away from the treated area (Roberts et al. 1997). The mathematical framework and sequence of behavioral events for understanding repellent, irritant, and toxic properties of insecticides in relation to mosquito control has been proposed (Roberts et al. 2000). Others have suggested behavioral responses and killing functions must be assessed together using different vectors, insecticides, and experimental conditions (Chareonviriyaphap et al. 2001, Sungvornyothin et al. 2001, Kongmee et al. 2004). Behavioral responses by mosquitoes to insecticides are influenced by a variety of environmental and biological factors that can be simulated under controlled experimental conditions (Busvine 1964, Elliott 1964, 1972, Kaschet 1969, Drobozina et al. 1984, Bondareva et al. 1986, Sungvornyothin et al. 2001). Physiological and nutritional conditions of mosquitoes have been reported as key factors influencing escape movement from insecticide-treated surfaces (Roberts et al. 1984, Sungvornyothin et al. 2001). Therefore, the design of studies for accurate measurement of avoidance behavior should include test conditions that would occur in the natural life history of the mosquito.

In this study, behavioral responses to deltamethrin and cypermethrin were compared using mated parous, nulliparous, early bloodfed, late bloodfed, sugarfed, and unmated cohorts from Kanchanaburi and Chiangmai regions of Thailand. The results provide new information on how physiological and nutritional conditions of mosquitoes can influence avoidance behavior. Generally, parous, nulliparous, and unmated mosquitoes demonstrated a far greater irritant escape response than recently blood or sugarfed mosquitoes. No significant repellency was observed in any of the test populations, regardless of physiological states, at the chemical

concentrations used. This is in agreement with previous findings on noncontact repellency responses of *Ae. aegypti* (Kongmee et al. 2004).

Our study found that the use of parous mosquitoes may strongly bias behavioral response tests. Given the consistent response and large percentage of parous females that made a rapid escape from contact with the surfaces of insecticide-treated chambers and controls trials, parity appears to confer greater spontaneous flight activity or sensitivity to test conditions (physiochemical, physical, and visual cues), some of which may be unrelated to the presence of insecticide. Chronological age may have also been a factor in escape response. Parous mosquitoes were older (8-9 d) than the other cohorts (2-6 d) at the time of testing.

A variety of factors can influence and regulate insect behavior. *Aedes aegypti* is a diurnal species and behavioral periodicities generally follow a bimodal pattern, entrained by external environmental oscillations (photic or thermal). Nevertheless, this mosquito remains generally active throughout the diel photoperiod. Laboratory rearing conditions can influence activity patterns dramatically depending on entrainment by phase-setting cues during larval development and early adulthood (Taylor and Jones 1969). Unfortunately, such studies are few but remain critical to a better understanding of natural behavioral patterns of adult mosquitoes and disease transmission (Corbet and Smith 1974, Jones 1981). For example, ambient temperature and humidity can affect insect behavioral periodicities as either inhibitory or permissive factors (Muirhead-Thomson 1938). During our laboratory trials, environmental parameters were maintained within reasonable ranges so as not to unduly influence responses.

Hitchcock (1968) classified adult behavior and activity patterns of mosquitoes at various points following emergence based on reproductive states. Parous females, for example, may have activity patterns that are strikingly different from other biological states in the adult life cycle. The significant avoidance responses of nulliparous and unmated females compared to the relative inactivity of pre-gravid and sugarfed adults may also be driven by endogenously controlled circadian rhythms or activity related to mating and foraging. Insemination status did not appear to influence the escape response in our trials, although others have demonstrated copulation-induced changes in the patterns of flight activity periodicities in mosquitoes (Jones 1981). For example, inseminated, non-bloodfed (nulliparous) females had nearly complete suppression of flight activity, only recovering gradually over a period of 7-8 d; whereas, other experiments observed a more rapid recovery (Taylor and Jones 1969, Jones 1981).

In comparison, there was an obvious reduction in insecticide escape responses in blood-fed and sugar-fed cohorts. Irritability responses are greatly suppressed following a recent blood meal (Hecht et al. 1960, Homan and Eyraud 1961, Roberts et al. 1984, Sungvornyothin et al. 2001); whereas unfed mosquitoes can show more pronounced irritant behavior (Qutubuddin 1967). A reluctance to fly from treated surfaces may be largely the result of the physical burden (weight) associated with imbibing a full blood meal and the pre-gravid process of blood digestion and ovarian development. Jones (1981) observed blood-engorged mosquitoes with greatly reduced flight activity becoming active again on the third day when they were gravid. Late blood-fed mosquitoes used in our study were at an advanced stage of vitellogensis but not yet fully gravid. The apparent diminished escape activity seen in sugar-fed mosquitoes may be a consequence of reduced sensory reception on the receptor hairs of tarsal segments contaminated with dried sugar solution (Elliott and Ochoa-Aguirre 1974, Soliman and Cutkomp 2001). *Aedes aegypti* becomes less active and unresponsive to resource (host) or environmental cues immediately after imbibing sugar solution (Jones and Madhukar 1976, Feinsod and Spielman 1979). The poor escape response and reduction in spontaneous flight activity in both bloodfed and sugarfed females are comparable to *Anopheles minimus* under near equivalent test conditions (Sungvornyothin et al. 2001).

A high percent mortality was observed with mosquitoes that remained in cypermethrin- treated chambers, and particularly so with the *Chiangmai* strain (Table 3). Background physiological resistance to cypermethrin and deltamethrin were dissimilar for both strains, with *Chiangmai* exhibiting greater susceptibility to cypermethrin than *Kanchanaburi* (40% vs. 15% mortality, respectively), which may explain the difference in response between strains. This does not preclude some other factor that may have produced sufficient contact with treated surfaces to increase mortality in spite of the high level of resistance seen in susceptibility tests. Blood meals also appear to have had some protective value against deltamethrin as mortality in these cohorts was far less than seen with cypermethrin. In this study, we observed temperature and humidity may have played significant roles in escape response and mortality. For *Chiangmai*, excito-repellency tests with deltamethrin were generally performed earlier in the morning hours with lower temperatures (average 27°C) and higher relative humidity (average 75%) compared to those of cypermethrin (average 32°C and 50% RH), which may account for the higher mortality seen with cypermethrin. Many synthetic pyrethroids exhibit significant vapor repellent activity as temperatures increase (Chareonviriyaphap et al. 1997). For those tests performed at higher temperatures, a greater percent mortality might be expected. Epidemiologically, the rapid escape response of older, parous females is seen as important because this cohort has the greatest potential for disease transmission. However, despite an obvious aversion for remaining inside contact and control chambers, parous exit mortality was amongst the highest seen in either strain or insecticide tested. High percentage mortality in the face of excito-repellency has also been documented with *Anopheles quadrimaculatus* and DDT (Wilson et al. 1973).

Many chemicals, including organophosphates, carbamates, and synthetic pyrethroids have long been used in public health vector control programs (Reiter and Gubler 1997). By 1994, deltamethrin and cypermethrin were in common use as space sprays in Thailand for controlling household nuisance and vector mosquitoes, including *Ae.*

aegypti (Chareonviriyaphap et al. 1999). Both chemicals (particularly deltamethrin) have been used to attempt interruption of mosquito virus transmission in communities immediately following reports of dengue cases. The need for more effective residual formulations and application technology is an area of continued investigation for adult mosquito control. We strongly encourage others examining the use of residual insecticides to carefully document the behavioral responses of *Ae. aegypti* in the study design. However, as our investigation clearly shows, careful consideration must also be given to the nutritional and physiological conditions of mosquitoes used in the evaluation.

The poor sustainability of *Ae. aegypti* control methods in most areas of the world is well documented (Gubler and Clark 1994, Gubler 1997, Reiter and Gubler 1997). The predicable host-seeking activities, indoor resting habits, and strong predilection to feed on humans inside houses would presumably cause this species to be more easily controlled than most other species. However, the strong anthropophagic and endophilic behavior of *Ae. aegypti* has presented a huge challenge to vector control professionals to devise new or improved methods to effectively reduce mosquito populations and disease transmission risk (WHO 1999). Residual insecticides applied indoors using conventional portable ultra-low-volume devices, mist blowers, and thermal fogging machines can provide longer lasting control of adult *Aedes* (Pant et al. 1974, Sulaiman et al.1993, Reiter and Gubler 1997, Perich et al. 2001) compared to more conventional methods using space sprays. Dramatic reductions in the Breteau Index (number of positive *Aedes* containers per 100 houses) and *Aedes* larval densities were reported following indoor residual sprays of alphacypermethrin (1.5% S.C.) at 0.02 g/m^2 in Taiwan (Lien et al. 1993), and Pant et al. (1974) reported up to 7 months of effective residual control of *Ae. aegypti* indoor densities using fenitrothion applied by aerosol mist blower. Former antimalarial campaigns that relied heavily on indoor residual insecticide applications also documented the dramatic reduction of *Ae. aegypti* populations over time (Giglioli 1948, 1954, Brown and Pal 1971).

An understanding of behavioral avoidance by mosquitoes that can interfere with vector feeding and alter other behavioral patterns (e.g., oviposition site preference) of adult mosquitoes, must be considered when assessing the operational effect of insecticides on dengue suppression, remembering the primary measure of successful control should be reduction of disease transmission (case incidence), not simply the quantitative reduction of vector mosquito densities. Despite over a century of study, there remains much to understand about the biology and behavior of *Ae. aegypti* regarding dengue transmission. The behavioral responses of mosquito vectors to insecticides are relevant to a better understanding of the mechanisms that may influence transmission and support the rationale for current mosquito control activities and expenditures. We believe excito-repellency assays should be an integral component of any evaluation of an insecticide's full attributes and potential to abate disease transmission.

Acknowledgments

This research project was supported by the Thailand Research Fund (TRF), the Center of Agricultural and Biotechnology (CAB), and the Kasetsart University Research and Development Institute (KURDI), Thailand. We thank Bayer Crop Science and Ladda Company, Thailand, for providing insecticides for this research.

REFERENCES CITED

Bondareva, N.L., M.M. Artem'ev, and G.V. Gracheva. 1986. Susceptibility and irritability caused by insecticides to malaria mosquitoes in the USSR. Part 1. *Anopheles pulcherrimus*. Med. Parazitol. Parazit. Biol. 6: 52-55.

Brown, A.W.A. and R. Pal. 1971. *Insecticide resistance in arthropods*. 2nd ed. Monograph Ser. 38. WHO, Geneva.

Busvine, J.R. 1964. The significance of DDT-irritability tests on mosquitoes. Bull. Wld. Hlth. Org. 31: 645-656.

Chareonviriyaphap, T., B. Aum-Aung, and S. Ratanatham. 1999. Current insecticide resistance patterns in mosquito vectors in Thailand. Southeast Asian J. Trop. Med. Publ. Hlth. 30: 184-194.

Chareonviriyaphap, T., D.R. Roberts, R.G. Andre, H. Harlan, and M.J. Bangs. 1997. Pesticide avoidance behavior in *Anopheles albimanus* Wiedemann. J. Am. Mosq. Contr. Assoc. 13: 171-183.

Chareonviriyaphap T., S. Sungvornyothin, S. Ratanatham, and A. Prabaripai. 2001. Pesticide-induced behavioral responses of *Anopheles minimus*, a malaria vector in Thailand. J. Am. Mosq. Contr. Assoc. 17: 13-22.

Chareonviriyaphap, T., A. Prabaripai, and S. Sungvornyothin S. 2002. An improved excito-repellency for mosquito behavioral test. J. Vector Ecol. 27: 250-252.

Chareonviriyaphap, T., A. Prabaripai, and M.J. Bangs. 2004. Excito-repellency of deltamethrin on the malaria vectors, *Anopheles minimus, Anopheles dirus, Anopheles sawadwongporni*, and *Anopheles maculatus*, in Thailand. J. Am. Mosq. Contr. Assoc. 20: 45-54.

Coosemans, M.H. and S. Sales. 1977. Stage VI evaluation of five insecticide-OMS 1821, OMS-1825, and OMS-1988 against anopheline mosquitoes at the Soumousso Experimental Station, Bobo Dioulasso, Upper Volta. WHO/VBC/77.663.

Corbet, P.S. and S.M. Smith. 1974. Diel periodicities of landing of nulliparous and parous *Aedes aegypti* (L.) at Dar es Salaam, Tanzania (Diptera: Culicidae). Bull. Entomol. Res. 64: 111-121.

Davidson, G. 1953. Experiments on the effect of residual insecticides in houses against *Anopheles gambiae* and *Anopheles funestus*. Bull. Entomol. Res. 44: 231-255.

de Zulueta, J. 1964. Ethological changes in malaria vectors. A review of the situation in light of recent findings. Riv. Malar. 43: 29-36.

Drobozina, V.P., M.M Artemev, G.V. Kashaeva, and R.L. Kuznetsov. 1984. Susceptibility to insecticides (DDT and malathion) and irritability on contact with them of malaria

mosquitoes of natural populations in the Dagestan USSR. Medit. Parazitol. Parazit. Biol. 6:44-46.

Elliott, R. 1964. Studies on the kinetic response of mosquitoes to chemicals. Bull. Wld. Hlth. Org. 31: 657-667.

Elliott, R. 1972. The influence of vector behavior on malaria transmission. Am. J. Trop. Med. Hyg. 21: 755-763.

Elliott, R. and O. Ochoa-Aguirre. 1974. Overnight access to sugar and response to DDT in *Anopheles albimanus* Wied. Bull. Wld. Hlth. Org. 51: 311-313.

Feinsod, F.M. and A. Spielman. 1979. An olfactometer for measuring host-seeking behavior of female *Aedes aegypti* (Diptera: Culicidae). J. Med. Entomol. 15: 282-285.

Giglioli, G. 1948. An investigation of the house-frequenting habits of mosquitoes of the British Guiana coastland in relation to the use of DDT. Am. J. Trop. Med. 28: 43-70.

Giglioli, G. 1954. Residual effect of DDT in a controlled area of British Guiana tested by the continued release of *Anopheles darlingi* and *Aedes aegypti*: A practical technique for the standardized evaluation of over-all residual efficiency under field conditions. Trans. R. Soc. Trop. Med. Hyg. 48: 506-521.

Gubler, D.J. 1997. Dengue and dengue haemorrhagic fever: its history and resurgence as a global public health problem. In: D.J Gubler and G. Kuno (eds.). *Dengue and dengue haemorrhagic fever.* pp. 1- 22. CAB International, NY.

Gubler, D.J. and G.G. Clark. 1994. Community-based integrated control of *Aedes aegypti*: a brief overview of current programs. Am. J. Trop. Med. Hyg. 50 (suppl.): 50-60.

Hecht, O., O. Mancera, and A. Barrera. 1960. Relation of DDT-irritation threshold to knockdown of three species of anopheline mosquitoes. J. Ecol. Entomol. 53: 634-640.

Hitchcock, J.C. Jr. 1968. Age composition of a natural population of *Anopheles quadrimaculatus* Say (Diptera: Culicidae) in Maryland, U.S.A. J. Med. Entomol. 5: 125-134.

Homan, J. and M. Eyraud. 1961. Etude des facteurs physiologiques conditionant, chez les Anopheles, I'irritabilite au DDT. Riv. Malarol. 40: 219-242.

Jones, J.C. and B.V. Madhukar. 1976. Effects of sucrose on blood avidity in mosquitoes. J. Insect Physiol. 22: 357-360.

Jones, M.D.R. 1981. The programming of circadian flight-activity in relation to mating and the gonotrophic cycle in the mosquito, *Aedes aegypti*. Physiol. Entomol. 6: 307-313.Jones, M.D.R. and S.J.Gubbins. 1978. Changes in the circadian flight activity of the mosquito *Anopheles gambiae* in relation to insemination, feeding and oviposition. Physiol. Entomol. 3: 213-220.

Kaschet, A.H. 1969. Effects of temperature on the irritability of anopheline mosquitoes due to DDT and DDT analogues. WHO/VBC/87: 994.

Kennedy, J.S. 1947. The excitant and repellent effects on mosquitoes of sub-lethal contacts with DDT. Bull. Entomol. Res. 37: 593-607.

Kongmee, M., A. Prabaripai, P. Akaratanakul, M.J. Bangs, and T. Chareonviriyaphap. 2004. Behavioral responses of *Aedes aegypti* (Diptera: Culicidae) exposed to deltamethrin and possible implications for disease control. J. Med. Entomol. 41: 1055-1063.

Lal, H., S. Ginocchio, and E.J. Hawrylewicz. 1965. Effect of allethrin on feeding behavior of insects. Proc. Soc. Exp. Biol. Med. 120: 441-443.

Lien, J.C., T.H. Lin, and H.M. Huang. 1993. Dengue vector surveillance and control in Taiwan. Trop. Med. 35: 269-276.

Mantel, N. and W. Haenzel. 1959. Statistic aspects of the analysis of data from retrospective studies of diseases. J. Natl. Cancer Instit. 22: 719-748.

Mattingly, P.F. 1962. Mosquito behavior in relation to disease eradication programmes. Annu. Rev. Entomol. 7: 419-436.

Moore, C.G. 1977. Insecticide avoidance by ovipositing *Aedes aegypti*. Mosq. News 37: 291-293.

Muirhead-Thomson, R.C. 1938. The reactions of mosquitoes to temperature and humidity. Bull. Entomol. Res. 29: 125-140.

Muirhead-Thomson, R.C. 1960. The significance of irritability, behaviouristic avoidance and allied phenomena in malaria eradication. Bull. Wld. Hlth. Org. 22: 721-734.

Pant C.P., H.L. Mathis, M.J. Nelson, and B. Phanthumachinda. 1974. A large-scale field trial of ultra-low-volume fenitrothion applied by a portable mist blower for the control of Aedes aegypti. Bull. Wld. Hlth. Org. 51: 409-415.

Pell, J.K., M.A. Spinney, and K.J. Ward. 1989. Observations on the behavior of adult *Anopheles gambiae* encountering residual deposits of lambda-cyhalothrin compared with the other major classes of commercially available insecticides. p. 18. Fourth Annual Conference of the Society for Vector Ecology, European Region.

Perich, M.J., C. Sherman, R. Burge, E. Gill, M. Quintana, and R.A. Wirtz. 2001. Evaluation of the efficacy of lambda-cyhalothrin applied as ultra-low volume and thermal fog for emergency control of *Aedes aegypti* in Honduras. J. Am. Mosq. Contr. Assoc. 17: 221-224.

Quinones, M.L. and M.F. Suarez. 1989. Irritability to DDT of natural populations of the primary malaria vectors in Colombia. J. Am. Mosq. Contr. Assoc. 5: 56-59.

Qutubuddin, M. 1967. Irritability of *Anopheles pharoensis* Theobald to different insecticides as observed in laboratory experiment. Sudan Med. J. 5: 18-28.

Rawling, P. and G. Davidson. 1982. The dispersal and survival of *Anopheles culicifacies* Giles (Diptera: Culicidae) in a Sri Lanka village under malathion spraying. Bull. Entomol. Res. 72: 139-144.

Ree, H.I. and K.P. Loong. 1989. Irritability of *Anopheles farauti, Anopheles maculatus*, and *Culex quinquefasciatus* to permethrin. Japan. J. San. Zool. 40: 47-51.

Reiter, P. and D.J. Gubler. 1997. Surveillance and control of urban dengue vectors. In: D.J. Gubler and G. Kuno (eds.), *Dengue and dengue haemorrhagic fever*. pp. 425-462.

CAB International, NY.

Roberts, D.R., W.D. Alecrim, A.M. Tavares, and K.M. Mc Neil. 1984. Influence of physiological condition on the behavioral response of *Anopheles darlingi* to DDT. Mosq. News 4: 357-561.

Roberts, D.R. and W.D. Alecrim. 1991. Behavioral response of *Anopheles darlingi* to DDT sprayed house walls in Amazonia. Pan Am. Hlth. Org. Bull. 25: 210-217.

Roberts, D.R. and R.G. Andre. 1994. Insecticide resistance issues in vector borne disease control. Am. J. Trop. Med. Hyg. 50: 21-34.

Roberts, D.R., T. Chareonviriyaphap, H.H. Harlan, and P. Hshieh. 1997. Methods for testing and analyzing excito-repellency responses of malaria vectors to insecticides. J. Am. Mosq. Contr. Assoc. 13: 13-17.

Roberts, D.R., W.D. Alecrim, P. Hshieh, J. Grieco, M.J. Bangs, R.G. Andre, and T. Chareonviriyaphap. 2000. A probability model of vector behavior: effects of DDT repellency, irritability, and toxicity in malaria control. J. Vector Ecol. 25: 48-61.

Rutledge, L.C., N.M. Echana, and R.K. Gupta. 1999. Responses of male and female mosquitoes to repellents in the World Health Organization insecticide irritability test system. J. Am. Mosq. Contr. Assoc. 15: 60-64.

Soliman, S.A. and L.K. Cutkomp. 2001. A comparison of chemoreceptor and whole fly responses to DDT and parathion. J. Econ. Entomol. 56: 492-494.

Somboon, P., L. Prapanthadara, and W. Suwonkerd. 2003. Insecticide susceptibility tests of *Anopheles minimus*, *Aedes aegypti*, *Aedes albopictus* and *Culex quinquefasciatus* in northern Thailand. Southeast Asian J. Trop. Med. Publ. Hlth. 34: 87-93.

Sulaiman, S., M.A. Karim, B. Omar, J. Jeffery, and F. Mansor. 1993. The residual effects of the synthetic pyrethroids lambda-cyhalothrin and cyfluthrin against *Aedes aegypti* (L.) in wooden huts in Malaysia. Mosq. Borne Dis. Bull. 10: 128-131.

Sungvornyothin, S., T. Chareonviriyaphap, A. Prabaripai, T. Trirakhupt, S. Ratanatham, and M.J. Bangs. 2001. Effects of nutritional and physiological status on behavioral avoidance of *Anopheles minimus* (Diptera: Culicidae) to DDT, deltamethrin and lambdacyhalothrin. J. Vector Ecol. 26: 202-215.

Taylor, B. and M.D.R. Jones. 1969. The circadian rhythm of flight activity in the mosquito *Aedes aegypti* (L.): the phase setting effects of light-on and light-off. J. Exp. Biol. 51: 59-70.

Threlkeld, S.F.H. 1985. Behavioral responses in *Drosophila melanogaster* associated with permethrin and ectiban. In: *Proceedings of 32nd Annual Meeting, Canadian Pest Management Society*. pp. 29-36. Canadian Pest Management Society, Charlottetown, Prince Island, Canada.

Wilson, H.G., D.E. Weidhaas, and G.C. LaBrecque. 1973. Toxicity of insecticide residues to *Anopheles quadrimaculatus* and effects on resting behavior. Mosq. News. 33: 539-542.

World Health Organization. 1992. Vector resistance to pesticides. Tech. Rep. Ser. 818. WHO, Geneva.

World Health Organization. 1996. Report of the WHO Informal Consultation on the evaluation and testing of insecticides. CTD/WHOPES/IC/96.1. Geneva, Switzerland.

World Health Organization. 1998. Report of WHO Informal Consultation. Test procedures for insecticide resistance monitoring in malaria vectors.

World Health Organization. 1999. Prevention and control and dengue and dengue haemorrhagic fever: Comprehensive guidelines. WHO Regional Publication, SEARO No. 29., New Delhi.

The effect of host type on movement patterns of *Aedes aegypti* (Diptera: Culicidae) into and out of experimental huts in Thailand

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Received 6 March 2006; Accepted 30 June 2006

ABSTRACT**:** Flight behavior studies were carried out from December 2004 through February 2005 at two sites in Thailand to compare the movement patterns of *Aedes aegypti* into and out of experimental huts baited with a human host, dog host, or without a host using a mark-release-recapture study design. Studies were conducted in isolated villages of Kanchanaburi and Chiang Mai Provinces, Thailand. In the presence of a human host only 4.9% (39/800) of the *Ae. aegypti* females departed the hut as compared to 46.5% (372/800) when a dog was present. There was no significant difference in the numbers of *Ae. aegypti* exiting when comparing dog to no host. A peak in exiting behavior in the absence of any host (human or dog) was observed between 1400-1700 h. Ingress behavior was much stronger when a human host was present in the hut with the peak of entering occurring in the morning (0830-1130 h) compared to 1000-1200 h without a host. Overall, significant differences between the two host types were observed with *Ae. aegypti* females being more attracted to humans *($p< 0.05$)* than dogs. There was no significant difference between numbers of *Ae. aegypti* entering the hut baited with a dog and the hut containing no host source. The experimental hut design used in the present study can serve as a protocol for testing the exiting and entering behavior of *Ae. aegypti* in response to chemical compounds. ***Journal of Vector Ecology*** **31 (2): 311-318. 2006.**

Keyword Index: Aedes aegypti, mosquitoes, exiting/entering movement, host type, Thailand.

INTRODUCTION

Several countries continue to experience endemic and re-emerging dengue fever (DF) and dengue hemorrhagic fever (DHF) (Gubler 1997). In Thailand, outbreaks of DHF were first recognized in Bangkok in 1957 and subsequently the disease has expanded throughout the country (Sheparrd et al. 1969, Chareonsook et al. 1996). In spite of continued vigilance in control measures, dengue cases in Thailand recently increased from 2000 to 2004. The reason for this increase is unclear but is most likely due to a combination of factors including the increase of human and economic activities in the urban and semi-urban zones. In addition, traditional water storage practices increase the availability of breeding sites for *Aedes aegypti*, a primary vector of DF and DHF (Kittayapong and Strickman 1993).

Aedes aegypti, a day biting mosquito, is highly anthropophilic and often resides in and near human dwellings (Christophers 1960, Yasuno and Tonn 1970, Gubler 1997, Thavara et al. 2001). This mosquito has been found to be highly adapted to all man-made and natural environments and is an extremely efficient vector of dengue (Chareonviriyaphap et al. 2003, Vazeille et al. 2003, Rodhain and Rosen 1997). Preventive measures for dengue rely entirely on vector control, the most effective method for reducing disease transmission in urban and semi-urban areas of the world (Reiter and Gubler 1997, Pant et al. 1974, Perich et al. 2001). Chemical control, however, is becoming increasingly difficult due to a number of issues including environmental concerns, international bans, adverse health effects, and insecticide resistance. For these reasons, a renewed effort is underway to identify novel compounds for use against the adult stage of this extremely efficient vector species. Many investigators have utilized experimental huts to study the ingress and egress behaviors of malaria vectors in response to insecticides applied to the interior surfaces of house walls (Kennedy 1947, Roberts and Alecrim 1991, Grieco et al. 2000, Chareonviriyaphap et al. 1997, 2001, Roberts et al. 2000). However, there is no standard protocol for evaluating test compounds using experimental huts with *Ae. aegypti* mosquitoes. This investigation was the first attempt to study the movement patterns of *Ae. aegypti* using a mark-release-recapture design and experimental huts baited with different host types; human, dog, and no host. The objectives of this study were to describe the movement patterns of *Ae. aegypti* into and out of experimental huts in response to different host stimuli and determine the relative attractiveness of huts baited with human hosts, dog hosts, or no hosts to blood-seeking females.

MATERIALS AND METHODS

Study sites

Two study sites located in Chiang Mai and Kanchanaburi were selected based on the criteria of high adult densities of *Ae*. *aegypti* and no recent evidence of local dengue transmission (Figure 1). Before beginning the study, both sites were mapped using a hand-held global positioning system (GPS) unit (Garmin International Inc., Olathe, KS) to ensure no indigenous homes were located within an 800 m diameter buffer zone around the experimental huts. Due to the short flight distance of this species (Harrington et al. 2005, Tsuda et al. 2001), this design reduced the potential for released *Ae. aegypti* flying to occupied homes. The first study site was located in Pu Teuy Village, TriYok District, Kanchanaburi Province (14°17' N, 99°11 E) and is approximately 100 km northwest of Bangkok. The second study site was located in Pang Mai Deang Village, Mae Taeng District, Chiang Mai Province (14°18 N, 98°12 E) which is located approximately 60 km north of Chiang Mai. At each study site, two identical experimental huts were positioned 100 m apart in an isolated area surrounded primarily by mountainous terrain and agricultural fields. All experimental huts had three windows and one door which could be affixed with window and door traps.

Mosquito populations

Two *Ae. aegypti* populations, Kanchanaburi and Chiang Mai, were collected as larvae and pupae at the villages from which the studies were conducted in September 2004. After the initial collection, weekly *Aedes* collections were undertaken to maintain a natural (i.e., field-based) genetic flow into the laboratory reared population. The colonies were maintained in the insectary at the Department of Entomology, Kasetsart University, Bangkok, and in the Office of Disease Control No. 10, Ministry of Public Health, Chiang Mai, Thailand.

Mosquito rearing and conditioning

Both *Ae. aegypti* colonies were reared following the method of Chareonviriyaphap et al. (1997). All life stages were maintained under controlled conditions (25$\pm$5° C and 80$\pm$10% relative humidity). Adults were maintained in 30 x 30 x 30 cm screened cages and were provided cotton pads soaked with a 10% sugar solution from the day of emergence. Female mosquitoes were provided a hamster blood meal on the fourth day post-emergence. Two days post blood feeding, oviposition dishes with filter paper were placed in the cage with the gravid females for collecting eggs.

Experimental huts

Four experimental huts were used in the study. Two huts were constructed at the Kanchanaburi field site and the other two were built at the Chiang Mai study site. All huts were constructed with identical materials and design. Hut walls were made from wood planks and the roof from zinc panels. The dimensions of the huts were 4 x 5 x 4 m with three movable entrance traps (window) and one exit trap (door). Entrance window traps were moved forward and backward during the observation period by sliding them on an aluminum support platform. Exit trap was affixed to the door of the experimental hut only when the exiting behavior was performed. Details for both hut construction and trap design have been published previously (Achee et al. 2005, Chareonviriyaphap et al. 2005).

Mosquito marking and release

Only the F1 adult generation was used in the study. At each site, two groups of 100 3 to 5-day-old non-blood-fed females were marked with a unique color of fluorescent dye following the method of Tsuda et al. (2001). Marked specimens were sugar-starved for 24 h, maintained in containers, and provided with water-soaked cotton pads until the time of release. Released populations were transferred to the field sites in Styrofoam ice chests. For the exiting behavior studies, marked females were released inside both huts 1 h prior to the start of the collection. For entering behavior studies, 100 marked mosquitoes were released 10 m outside of each hut. All mosquitoes were released at 0500 h and recapture collections were performed from 0600 to 1800 h. The study was carried out from December 2004 to February 2005.

Recapture collection

All experiments were replicated two times at each hut and at each location. In the presence of a host, the human or dog was placed under a mosquito net to protect them from biting mosquitoes. Exit and entrance traps were sampled at 20 min intervals by three collectors per hut during a 12-h collection period (0600-1800 h). Mosquitoes collected from the traps were placed into holding cages labeled with the location and time of collection. To control for collector bias, collectors were rotated between huts every 6 h. All mosquitoes from the traps were examined for fluorescent marking using UV illumination under a dissecting microscope. When mosquitoes were released inside the hut, backpack aspiration of the interior of the huts was performed at the end of each 12 h collection period to ensure that all remaining mosquitoes were recaptured to be ready for the next experiment.

Data analysis

Mean numbers of recaptured mosquitoes were analyzed by a three-way analysis of variance (ANOVA) with three types of host designs with two huts nested within each type. Location served as a block. Fisher's Least Significant Differences (LSD) was used to compare the difference in ingress and egress movement by host. All analyses were performed using SPSS 12.0 version (SPSS 2003, Cary, NC, U.S.A.). The discriminating level for all significance tests was 0.05%.

RESULTS

Numbers of *Ae. aegypti* females recaptured from entrance and exit traps from both study sites are presented in Tables 1 and 2, respectively. Between field sites there were no statistical differences in the total numbers of recaptured females exiting (F_3=0.692; P=0.569) and entering (F_3=1.178; P=0.348) the huts. Therefore, data from both study locations were pooled for further analysis. Differences were found in the number of *Ae. aegypti* exiting the hut (F_2=31.588; P=0.000) and in the entrance traps (F_2=8.447; P=0.003) when comparing the three host conditions. The number of *Ae. aegypti* recaptured from entrance traps when baited with a human host (166) was greater than dog-baited huts (87; *P=0.006*) or huts with no host (69; *P=0.001*). There was no difference in total number of recaptured females collected from entrance traps affixed to huts containing a dog host (87) or an empty hut (69; *P=0.448*) (Table1). Overall, 20.8% of females were recaptured in the entrance traps in the presence of a human host, whereas only 10.9% and 8.6% were recaptured in the entrance traps when a dog or no host was used, respectively (Table 1).

Analyses of exit trap collections indicate there was a significant increase in the number of *Ae. aegypti* exiting the huts when a dog host (372) was present compared to a human host (39; *P<0.001*). However, there was no difference between the numbers recaptured in exit traps affixed to huts containing a dog host (372) compared to an empty hut (364; *P=0.983*). Overall, only 4.9% of *Ae. aegypti* females departed a hut when a human served as a host compared to 46.5% and 45.5% with dog-baited or no host inside the huts, respectively (Table 2).

Time trends for entering *Ae. aegypti* was also evaluated in response to the three different host conditions (Figure 2). The average number of females entering the hut was highest at each collection hour when a human host was present; although, the time when marked females began entering the hut (0700 h) was the same as when a dog was present. When no host was present, marked females did not begin to enter the hut until 1000 h, three hours later than when a vertebrate host, dog or human, was available. Additionally, the peak time of recapture (1100 h) was similar between huts containing a dog or no host.

Time trends of exiting behavior indicated minimum exiting during the entire collection period when a human host was inside the hut compared to when a dog or no host were present (Figure 3). Even though the total numbers exiting the hut were highest when a host was absent, the time period when females began to exit from the hut (1100 h) was similar as when a dog host was present. The peak time of exiting was the same whether a dog or no host was present.

DISCUSSION

A mark-release-recapture study design was used in conjunction with an experimental hut fitted with entrance and exit traps to evaluate the relative attractiveness and flight response of *Aedes aegypti* to different host types. In this study, *Ae. aegypti* exhibited a higher attraction for huts containing human hosts compared to huts with either dogs or no host. Huts baited with dogs or without a host resulted in significantly fewer mosquitoes moving into the hut compared to when a human host was present. Huts baited with a dog were no more attractive to host-seeking female *Ae. aegypti* than huts containing no host at all. In addition, the majority of *Ae. aegypti* females remained in the hut as long as there was a human present. This is a clear indication that as hosts, dogs are not as attractive as humans. This is not surprising considering this species' natural history shows it to be highly anthropophilic. Previous studies on host preference suggested that *Ae. aegypti* is more likely to feed on human blood even when other animal hosts are freely available (Xue et al. 1995, Harrington et al. 2001, Polawat and Harrington 2005, Christophers 1960, Chow et al. 1993, Edman et al. 1992, Templis 1975, Scott et al. 1993, 2000).

The time at which marked *Ae. aegypti* began to enter the hut when a host was present, dog or human, was approximately 3 h post-release, nearly 2 h earlier than when there was no host present in the hut. This is clearly a reflection of *Ae. aegypti*'s strong endophagic behavior. In addition, the complex interaction of host factors, as well as the physiological and nutritional status of the host-seeking female mosquito, may influence the entering flight behavior of *Ae. aegypti*. The movement of mosquitoes into the hut when a host was not present was most likely due to environmental pressures of the outdoor environment and the desire to seek shelter. When the outdoor environment experiences an increase in ambient temperature accompanied by a decrease in humidity during the increasing late morning hours, this appears to force female *Ae. aegypti* to seek suitable resting sites inside the protective environment of the hut.

In this study, the movement patterns for natural populations of *Ae. aegypti* into and out of huts in the presence of a human host are similar to those reported by Chareonviriyaphap et al. (2005). However, the exiting trend in this present study is slightly different from our previous observations. One of the major factors was likely the result of environmental pressure, especially increasing outdoor temperatures. The study by Chareonviriyaphap et al. (2005) was conducted mostly during the rainy period, whereas the current study was done mostly during the seasonably dry and cooler period of the year. The effect of temperature on movement patterns of *Ae. aegypti* will be under further investigation.

Previous research has suggested that residual insecticides applied indoors can provide longer-lasting control of adult *Aedes* (Sulaiman et al. 1993, Reiter and Gubler 1997). Malaria control programs that have relied heavily on the application of indoor residual insecticides have also documented a significant reduction in *Ae. aegypti* populations (Giglioli 1948). Studies with anopheline vectors have documented this reduction to be partially

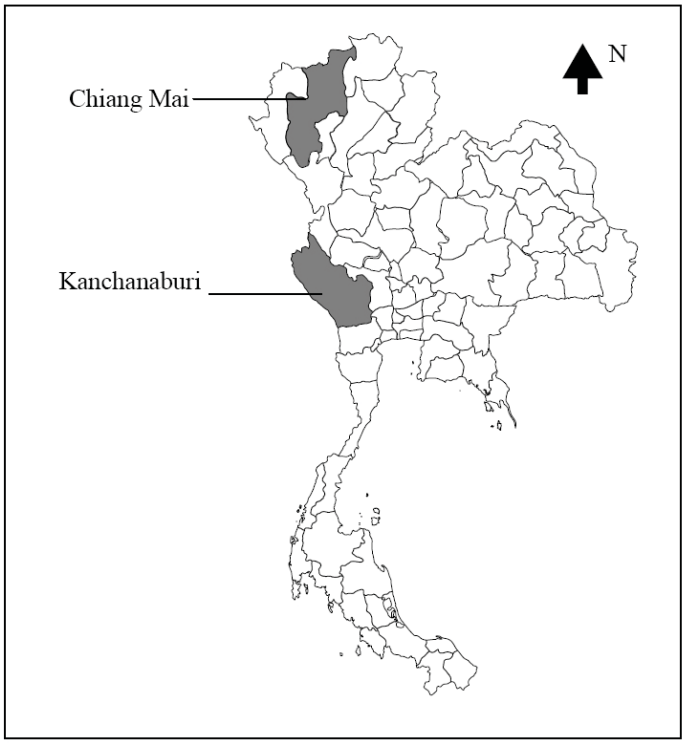

Figure 1. Map of Thailand indicating study sites, Kanchanaburi and Chiang Mai.

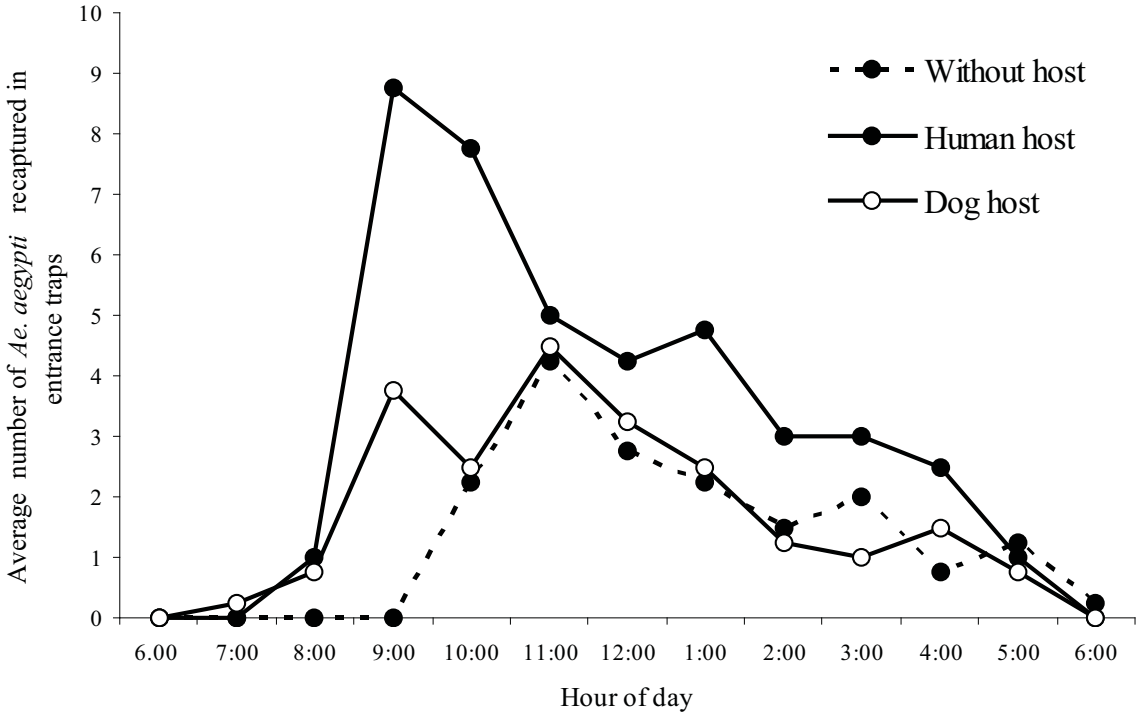

Figure 2. Time of entry of marked *Aedes aegypti* females into experimental huts during a 12 h sampling period.

Table 1. Number of marked *Ae. aegypti* females recaptured from entrance traps during two days of collections in the presence and absence of hosts.

Sites	Huts/ Replicates	Human host		Dog host		No host	
		Number Released	Recaptured in traps[1]	Number Released	Recaptured in traps[1]	Number Released	Recaptured in traps[1]
CM	1	100	24	100	9	100	10
	2	100	20	100	18	100	14
	1	100	32	100	10	100	12
	2	100	22	100	12	100	11
	Total (%)	400	98 (24.5) a[2]	400	49 (12.3) b[2]	400	47 (11.8) b[2]
Kan	1	100	2	100	6	100	5
	2	100	15	100	8	100	6
	1	100	23	100	6	100	3
	2	100	28	100	18	100	8
	Total (%)	400	68 (17) a[2]	400	38 (9.5) b[2]	400	22 (5.5) b[2]
	Total	800	166 (20.8) a[2]	800	87 (10.9) b[2]	800	69 (8.6) b[2]

CM: Chiang Mai; Kan: Kanchanaburi.
[1]3 traps on windows. [2]The same lowercase letter designates no significant difference at $p < 0.05$.

Table 2. Number of marked *Ae. aegypti* females recaptured from an exit trap affixed to the door of experimental huts during two days of collections in the presence and absence of hosts.

Sites	Huts/ Replicates	Human host		Dog host		No host	
		Number Released	Recaptured in traps[1]	Number Released	Recaptured in traps[1]	Number Released	Recaptured in traps[1]
CM	1	100	3	100	49	100	43
	2	100	17	100	67	100	33
	1	100	0	100	35	100	22
	2	100	4	100	34	100	25
	Total (%)	400	24 (6) a[2]	400	185 (46.3) b[2]	400	123 (30.8) b[2]
Kan	1	100	8	100	56	100	59
	2	100	7	100	40	100	59
	1	100	0	100	30	100	70
	2	100	0	100	61	100	53
	Total (%)	400	15 (3.8) a[2]	400	187 (46.8) b[2]	400	241 (60.3) b[2]
	Total (%)	800	39 (4.9) a[2]	800	372 (46.5) b[2]	800	364 (45.5) b[2]

CM: Chiang Mai, Kan: Kanchanaburi.
[1]One trap on door.
[2]The same lowercase letter designates no significant difference at $p < 0.05$.

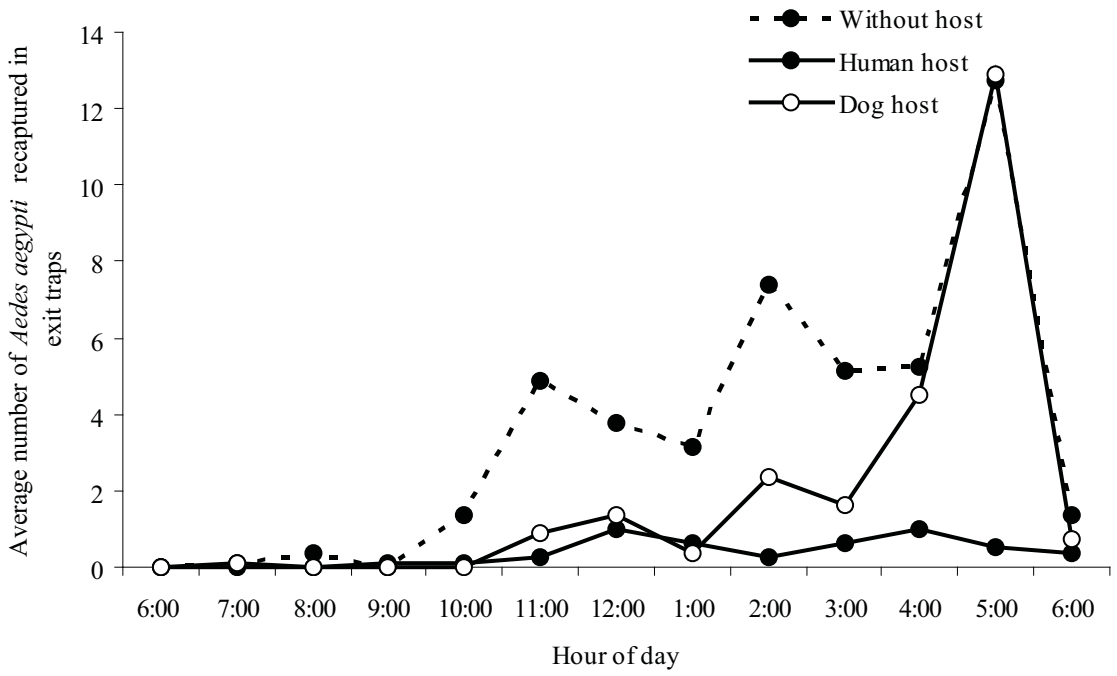

Figure 3. Time of exit of marked *Aedes aegypti* females from experimental huts during a 12 h sampling period.

due to the repellent and irritant actions of the chemicals influencing mosquito avoidance behavior (Grieco et al. 2000). For this reason, it is critical to access the potential behavioral modifying effects these compounds have on *Ae. aegypti* populations. The results of this study suggest that in order to more accurately access the behavioral responses to compounds, studies should place humans in sprayed huts to serve as attractive bait. This information will be useful for designing future studies examining the exiting and entering behaviors of *Ae. aegypti* in response to chemical compounds. Use of indoor residual spray with various test compounds, concentrations, and formulations in the attempt to reduce dengue transmission will be the subject of further study.

Acknowledgments

We thank the Disease Control Department, Ministry of Public Health, Nontaburi Province and Vector Borne Disease Section, Office of Disease Prevention and Control Department of Disease Control, Ministry of Public Health, Chiang Mai, Thailand, for the use of facilities during implementation of this work. We also thank the Armed Forces Development Command, Tri Yok District, Kanchanaburi Province and Head Village Office of Pang Mai Deang village, Mae Taeng District, Chiang Mai Province, for permission to use the study areas. Special thanks to Michael J. Bangs and the anonymous reviewers for their valuable comments, the students of the Department of Entomology, Faculty of Agriculture, Kasetsart University and the Chiang Mai entomological team, who helped with construction of huts and data collections. Funding for this research came from the National Institutes of Health (Grant # 5U01AI054777-02).

REFERENCES CITED

Achee, N., J.P. Grieco, R.G. Andre, E. Rejmankova, and D.R. Roberts. 2005. A mark-release-recapture study using a novel potable hut design to define the flight behavior of *Anopheles darlingi* in Belize, Central America. J. Am. Mosq. Contr. Assoc. 21: 366-379.

Chareonsook, O., H.M. Foy, A. Teerarakul, and N. Silarug. 1996. Changing epidemiology of dengue hemorrhagic fever in Thailand. Epidemiol. Infect. 122: 161–166.

Chareonviriyaphap, T., D.R. Roberts, R.G. Andre, H. Harlan, and M.J. Bangs. 1997. Pesticide avoidance behavior in *Anopheles albimanus*, a malaria vector in the Americas. J. Am. Mosq. Contr. Assoc. 13: 171-183.

Chareonviriyaphap, T., S. Sungvornyothin, S. Ratanatham, and A. Prabaripai. 2001. Insecticide-induced behavioral responses of *Anopheles minimus*, a malaria vector in Thailand. J. Am. Mosq. Contr. Assoc. 17: 13-22.

Chareonviriyaphap, T., P. Akratanakul, S. Nettanomsak, and S. Huntamai. 2003. Larval habitats and distribution patterns of *Aedes aegypti* (Linnaeus) and *Aedes albopictus* (Skuse), in Thailand. Southeast Asian J. Trop. Med. Publ. Hlth. 34: 529-535.

Chareonviriyaphap, T., W. Suwonkerd, P. Mongkalakoon, A. Nicole, J. Grieco, B. Farlow, and D.R. Roberts. 2005. The use of an experimental hut for evaluating

the entering and exiting behavior of *Aedes aegypti* (Diptera: Culicidae), a primary vector of dengue in Thailand. J. Vector Ecol. 30: 344-346.

Chow, E., R.A. Wirtz, and T.W. Scott. 1993. Identification of blood meals in *Aedes aegypti* by antibody sandwich enzyme-link immunosorbent assay. J. Am. Mosq. Contr. Assoc. 9: 196-205.

Christophers, S.R. 1960. *Aedes aegypti* (L.), *the Yellow Fever Mosquito*. Cambridge University Press, London.

Edman, J.D., D. Strickman, P. Kittayapong, and T.W. Scott. 1992. Female *Aedes aegypti* (Diptera: Culicidae) in Thailand rarely feed on sugar. J. Med. Entomol. 29: 1035–1038.

Giglioli, G. 1948. An investigation of the house frequenting habits of mosquitoes of the British Guiana coastland in relation to the use of DDT. Am. J. Trop. Med. Hyg. 28: 43-70.

Grieco, J., N. Achee, R.G. Andre, and D.R. Roberts. 2000. A comparison study of house entering and exiting behavior of *Anopheles vestitipennis* (Diptera: Culicidae) using experimental huts sprayed with DDT or deltamethrin in southern District of Toledo, Belize, C.A. J. Vector Ecol. 25: 62-73.

Gubler, D.J. 1997. Dengue and dengue haemorrhagic fever: its history and resurgence as a global public health problem. In: D.J. Gulber and G. Kuno (eds.) *Dengue and Dengue Haemorrhagic Fever*. pp. 1-22.Wallingford, Oxon: CAB International.

Harrington, L.C., J.D. Edman, and T.W. Scott. 2001. Why do female *Aedes aegypti* (Diptera: Culicidae) feed preferentially and frequently on human blood? J. Med. Entomol. 38: 411–422.

Harrington, L.C., T.W. Scott, K. Lerdthusnee, R.C. Coleman, A. Costero, G.G. Clark, J. Jones, S. Kithawee, P. Kittiyapong, R. Sithiprasasna, and J.D. Edman. 2005. Dispersal of the dengue vector *Aedes aegypti* within and between rural communities. Am. J. Trop. Med. Hyg. 72: 209-220.

Kennedy, J.S. 1947. The excitant and repellent effects on mosquitoes of sub-lethal contact with DDT. Bull. Entomol. Res. 37: 593-607.

Kittayapong, P. and D. Strickman. 1993. Distribution of container in habiting *Aedes* larvae (Diptera: Culicidae) at a dengue focus in Thailand. J. Med. Entomol. 30: 601–606.

Pant, C.P., H.L. Mathis, M.J. Nelson, and B. Phanthumachinda. 1974. A large scale field trial of ultra-low-volume fenitrothion applied by a portable mist blower for the control of *Aedes aegypti.* Bull. Wld. Hlth. Org. 51: 409-415.

Perich, M.J., C. Sherman, R. Burge, E. Gill, M. Quintana, and R.A. Wirtz. 2001. Evaluation of efficacy of lambdacyhalothrin applied as ultra-low-volume and thermal for emergency control of *Aedes aegypti* in Honduras. J. Am. Mosq. Contr. Assoc. 17: 221-224.

Polawat, A. and L.C. Harrington. 2005. Blood feeding patterns of *Aedes aegypti* and *Aedes albopictus* in Thailand. J. Med. Entomol. 42: 844-849.

Reiter, P. and D.J. Gubler 1997. Surveillance and control of urban dengue vectors. In: D.J. Gubler and G. Kuno (eds.) *Dengue and dengue haemorrhagic fever.* pp. 425-462. CAB International.

Roberts, D.R. and W.D. Alecrim 1991. Behavioral response of *Anopheles darlingi* to DDT sprayed house walls in Amazonia. Pan Am. Hlth. Org. Bull. 25: 210-217.

Roberts, D.R., W.D. Alecrim, P. Hshieh, J. Grieco, M.J. Bangs, R.G. Andre, and T. Chareonviriyaphap. 2000. A probability model of vector behavior: effects of DDT repellency, irritability, and toxicity in malaria control. J. Vector Ecol. 25: 48-61.

Rodhain, F. and F. Rosen. Mosquito vectors and dengue virus-vector relationships. In: D.J. Gulber and G. Kuno. (eds.) *Dengue and dengue haemorrhagic fever*. pp. 45-60. CAB International.

Scott, T.W., E. Chow, E. Strickman, P. Kittayapong, R.A. Wirtz, L.H. Lorenz, and J.D. Edman. 1993. Blood feeding patterns of *Aedes aegypti* (Diptera: Culicidae) during a single gonotrophic cycle using a histological technique. J. Med. Entomol. 30: 922-927.

Scott, T.W., A. Naksathit, J.F. Day, P. Kittayapong, and J.D. Edman. 1997. A fitness advantage for *Aedes aegypti* and the viruses it transmits when females feed only on human blood. Am. J. Trop. Med. Hyg. 57: 235-239.

Scott, T.W., A.C. Morrison, L.H. Lorenz, G.G. Clark, D. Strickman, P. Kittiyapong, H. Zhou, and J.D. Edman. 2000. Longitudinal studies of *Aedes aegypti* (Diptera: Culicidae) in Thailand and Puerto Rico: Blood feeding frequency. J. Med. Entomol. 37: 89-101.

Sheppard, P.M., W.W. Macdonald, R.J. Tonn, and B. Grab. 1969. The dynamics of an adult population of *Aedes aegypti* in relation to DHF in Bangkok. J. Animal Ecol. 38: 661-702.

Somboon P., L. Prapanthadara, and W. Suwonkerd. 2003. Insecticide susceptibility tests of *Anopheles minimus, Aedes aegypti, Aedes albopictus*, and *Culex quinquefasciatus* in northern Thailand. Southeast Asian J. Trop. Med. Publ. Hlth. 34: 87-93.

Sulaiman, S., M.A. Karim, B. Omar, J. Jeffery, and F. Mansor. 1993. The residual effects of the synthetic pyrethroids lambdacyhalothrin and cyfluthrin against *Aedes aegypti* in wooden huts in Malaysia. Mosq. Borne Dis. Bull. 10: 128-131.

Templis, C.H. 1975. Host feeding patterns of mosquitoes, with a review of advances in analysis of blood meals by serology. J. Med. Entomol. 11: 635-653.

Thavara, U., A. Tawatsin, C. Chansang, W. Kong-ngamsuk, S. Paosriwong, J. Boon-Long, Y. Rongsriyam, and N. Komalamisra. 2001. Larval occurrence, oviposition behavior and biting activity of potential mosquito vectors of dengue on Samui Island, Thailand. J. Vector Ecol. 23: 172-180.

Trpis, M. and W. Hausermann. 1978. Genetics of house entering behavior in east African populations of *Aedes aegypti* and its relevance to speciation. Bull. Entomol. Res. 68: 521-532.

Tsuda, Y., M. Takagi, S. Wang, Z. Wang, and L. Tang. 2001. Movement of *Aedes aegypti* (Diptera: Culicidae) released in a small isolated village on Hainan Island, China. J. Med. Entomol. 38: 93-98.

Vazeille, M., L. Rosen, L. Mousson, and A. Failloux. 2003. Low oral receptivity for dengue type 2 viruses of *Aedes albopictus* from Southeast Asia compared with that of *Aedes aegypti*. Am. J. Trop. Med. Hyg. 68: 203-208.

Yasuno, M. and R.J. Tonn. 1970. A study of habits of *Aedes aegypti* in Bangkok, Thailand. Wld. Hlth. Org. Bull. 43: 319-325.

Xue, R., J.D. Edman, and T.W. Scott. 1995. Age and body size effects on blood meal size and multiple blood feeding by *Aedes aegypti* (Diptera: Culicidae). J. Med. Entomol. 32: 471-474.

Scientific Note

An automated, field-compatible device for excito-repellency assays in mosquitoes

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The behavioral responses to insecticides by mosquitoes are important components of a chemical's overall effectiveness in reducing human-vector contact and should be carefully evaluated to understand the primary mechanisms involved in controlling vector activity and disease transmission. Excito-repellency (ER) responses of mosquitoes, divided into contact irritancy and noncontact repellency to chemicals (Roberts et al. 1997), have been evaluated in a number of ways. One of the first methods was developed by the World Health Organization using plywood to construct an ER test box that measured the irritant effect of insecticides on mosquitoes, followed by studies using various modifications of the WHO test design (Bondareva et al. 1986, Ree and Loong 1989, Pell et al. 1989, Quinones and Suarez 1989). Rachou et al. (1966) developed a plywood experimental box for testing the escape responses of *Anopheles albimanus* to DDT, and a similar test system was also used to observe the behavioral responses of *Anopheles darlingi* to DDT exposure (Charlwood and Paraluppi 1978). Roberts et al. (1984) developed a collapsible excito-repellency test box for field testing of *An. darlingi* against DDT. Years later, a light proof test chamber was developed to study the behavioral responses of *Anopheles gambiae* to several test compounds (Evans 1993).

Because of the inherent complexities of accurately measuring excito-repellency in mosquitoes, no one test method had been widely accepted as a standard for conducting assays, data gathering, analysis, and interpretation (Brown 1964, Roberts et al. 1984). Improvement came when an experimental escape chamber system was developed that could distinguish irritancy and repellency (Roberts et al. 1997). This test system was first used to study the avoidance behavior of *An. albimanus* to DDT and synthetic pyrethroids in Central America (Chareonviriyaphap et al. 1997). However, it proved to be somewhat cumbersome and required extended time to set up and attach test papers (treated and untreated) on the inside walls of the chambers. Soon afterwards, another version of the excito-repellency test chamber was devised to help alleviate some the burdens associated with the previous test design (Chareonviriyaphap and Aum-Aung 2000, Chareonviriyaphap et al. 2002) and proved valuable in the evaluation of behavioral responses by several laboratory and field populations of mosquitoes in Thailand and Indonesia (Chareonviriyaphap et al. 2001, 2004, Sungvornyothin et al. 2001, Kongmee et al. 2004, Potikasikorn et al. 2005, Chareonviriyaphap et al. 2006). Unlike previous "fixed" construction designs, the new chamber system was a collapsible device for easier transport to the field, and it also greatly reduced the time required to attach the test papers between test trials. However, this system was still cumbersome and required a minimum of two investigators to observe and record data during the 30-min testing period. The test design also required a relatively high number of mosquitoes (25 per test chamber), at times an impractical demand under field conditions. Recently, an assay for evaluating excito-repellency and toxicity in adult mosquitoes was developed (Grieco et al. 2006); but it was not designed as a field-adaptable apparatus. To help overcome this frequent problem when conducting field studies, a more compatible design has evolved. For the device described here, two major modifications from previous models were made: a substantial reduction in the size of the test box and the use of an electronic sensor for automated counting of mosquitoes as they escaped from the test chamber through the opening gate into the external holding cage (Figure 1).

The fundamental structural design of the new ER chamber (1) remains similar to the previous version (Chareonviriyaphap et al. 2002). The main supporting structure is fabricated using stainless steel, each side wall measuring 23 x 23 cm^2 in size. The chamber walls have an aluminum side tongue and groove configuration on joining ends that makes it easier and faster to set up and disassemble for transportation and storage. The frame of the inner chamber is constructed of 22.5 x 19 cm stainless steal beams, which include metal holders for securing test papers on either of two sides for the dual purpose of either providing contact or noncontact exposure designs. For noncontact tests, a thin sheet of fine mesh iron screening secured on the opposite side of the test paper allows for a 1.5 cm gap that prevents mosquito tarsal contact with the test paper. A Plexiglas™ panel at the rear of the chamber is equipped with a 11.5 cm diameter hole sealed with overlapping dental dam, allowing test specimens to be either inserted or removed from the inside of the chamber while minimizing accidental escape during handling. There is a forward exit portal (13.5 cm x 2 cm) connected to a funnel projecting from

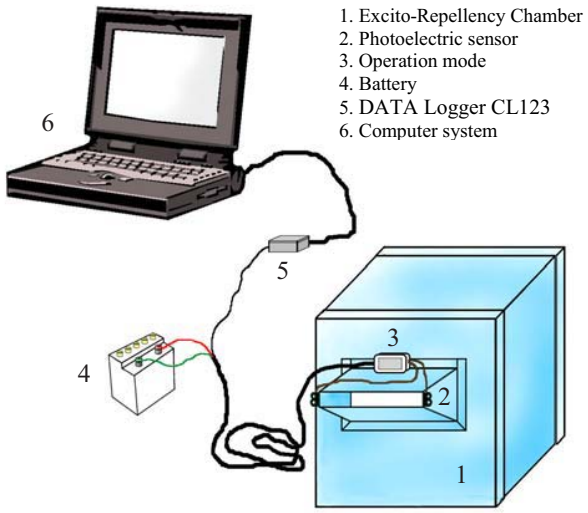

Figure 1. Automated excito-repellency test system.

the box with an electronic sensing device affixed at the point of the exit portal.

The photoelectric sensor (FX-301, SUNX Limited, Aichi, Japan) (Figure 1, #2) detects and counts escaping mosquitoes, automatically recording any flying object < 0.1 mm in size without requiring physical contact. The sensor has two operation mode switches (#3), a jog switch, and a MODE key required for operating the system. The MODE key operates the "mode selection" and "mode cancel" functions, while the jog switch selects the desired numerical values available for each mode. To record data during the observation period, the DATA Logger CL123 (#5) is connected to the photoelectric sensor and records values at three signal channels, one analog and two digital. The DATA Logger CL123 is a small, battery-operated device (#4) with software to record and transfer data in tabular and graphic form to the computer system (#6). The entire system can be programmed to record escaping mosquitoes at 30 s-intervals until test completion (30 min). The previous recording interval with human observation was set at 1-min periods.

This improved system provides distinct advantages over the previous version as it can accurately and automatically count and record escaping mosquitoes, thereby eliminating error by human observation alone while also preventing any possible confounding factors or bias produced by human attractant/stimulant cues (e.g., carbon-dioxide, odor, body heat) that could influence test results. The reduction in size of the device also makes it easier to transport to and from the field.

This improved excito-repellency device has been used to measure the behavioral responses of a field population of *Aedes aegypti* from Bangkok to single standard operational field concentrations of 0.02 g/m^2 of deltamethrin. Assay results revealed that test mosquito populations quickly departed chambers, indicating strong irritancy following direct contact with deltamethrin (Figure 2). As in previous studies, a complete test trial consists of four chambers, two treated with insecticides (one for contact, the other for noncontact) and two paired control (without treatment) chambers, respectively. However, we have reduced the number of unfed female mosquitoes required for each chamber from 25 to 15, a 40% reduction per trial, while retaining the statistical accuracy of the analysis (Roberts et al. 1997). This improved test chamber provides a highly reliable and objective record of the precise

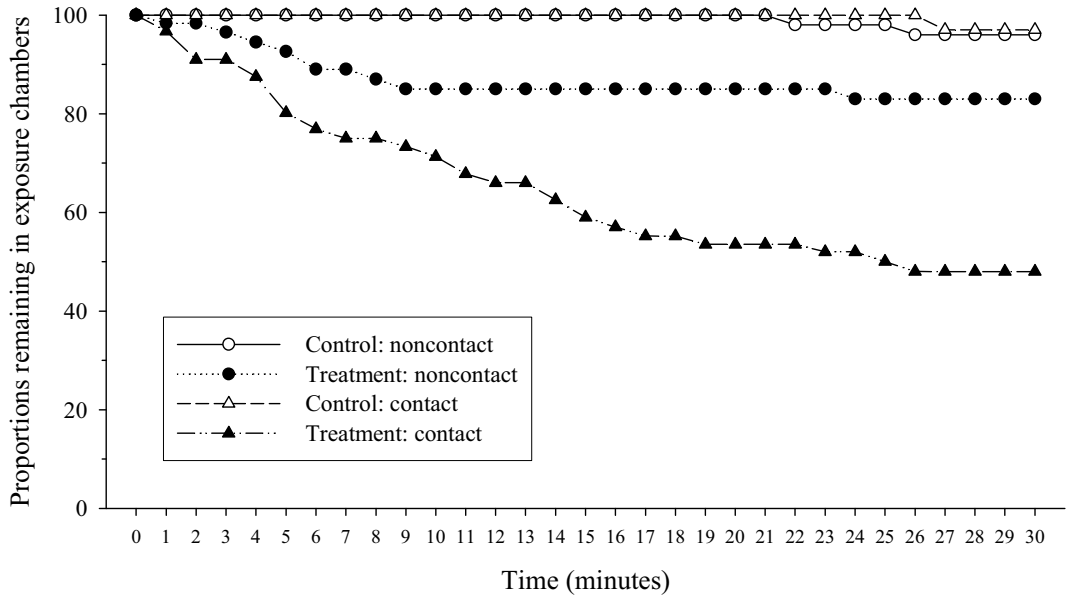

Figure 2. Behavioral responses of *Aedes aegypti* exposed to deltamethrin (0.02 g/m^2) in contact and noncontact exposures.

time interval when mosquitoes exit the test chambers. The design retains the ability to be easily transported to the field and, together with a substantial reduction in the previous number of mosquitoes required per test and the automated counting of exiting mosquitoes using a photoelectric sensor, allows greater flexibility to conduct excito-repellency tests. This automated detection system is easy to operate and eliminates human observer error.

Acknowledgments

The authors thank Dr. Michael J. Bangs for the critical review of this manuscript. This project was funded by the Thailand Research Fund and the Kasetsart University and Research Development Institute, Kasetsart University, Bangkok, Thailand.

REFERENCES CITED

Bondareva, N.L., M.M. Artem'ev, and G.V. Gracheva. 1986. Susceptibility and irritability caused by insecticides to malaria mosquitoes in the USSR. Part 1. *Anopheles pulcherrimus*. Meditsinskaia Parazitologha I Parazitarnye Bolezni (Moskva) 6: 52-55.

Brown, A.W.A. 1964. Experimental observations governing the choice of test method for determining the DDT-irritability of adult mosquitoes. Bull. Wld. Hlth. Org. 30: 97-111.

Chareonviriyaphap, T., D.R. Roberts, R.G. Andre, H. Harlan, and M.J. Bangs. 1997. Pesticide avoidance behavior in *Anopheles albimanus* Wiedemann. J. Am. Mosq. Contr. Assoc. 13: 171-183.

Chareonviriyaphap, T. and B. Aum-Aung 2000. Improved excito-repellency escape chamber for behavioral tests on mosquitoes. Mekong Malaria Forum 5: 82-86.

Chareonviriyaphap, T., S. Sungvornyothin, S. Ratanatham, and A. Prabaripai. 2001. Pesticide-induced behavioral responses of *Anopheles minimus*, a malaria vector in Thailand. J. Am. Mosq. Contr. Assoc. 17: 13-22.

Chareonviriyaphap, T., A. Prabaripai, and S. Sungvornyothin. 2002. An improved excito-repellency for mosquito behavioral test. J. Vector Ecol. 27: 250-252.

Chareonviriyaphap, T., A. Prabaripai, and M.J. Bangs. 2004. Excito-repellency of deltamethrin on the malaria vectors, *Anopheles minimus, Anopheles dirus, Anopheles swadiwongporni*, and *Anopheles maculatus*, in Thailand. J. Am. Mosq. Contr. Assoc. 20: 45-54.

Chareonviriyaphap, T., M. Kongmee, M.J. Bangs, S. Sathantriphop, V. Meunworn, A. Prabaripai, W. Suwonkerd, and P. Akratanakul. 2006. Influence of nutritional and physiological status on behavioral responses of *Aedes aegypti* (Diptera: Culicidae) to deltamethrin and cypermethrin. J. Vector Ecol. 31: 90-102.

Charlwood, J.D. and N.D. Paraluppi. 1978. The use of excito-repellency boxes with *Anopheles darlingi* Root, *An. nuneztovari* Gabaldon and *Culex pipiens quinquefasciatus* Say, obtained from the areas near Manaus, Amazonas. Acta Amazonica 8: 605-611.

Evans, R.G. 1993. Laboratory evaluation of the irritancy of bendiocarb, lambdacyhalothrin, and DDT to *Anopheles gambiae*. J. Am. Mosq. Contr. Assoc. 9: 285-293.

Grieco, J.P., N.L. Achee, M.R. Sardelis, K.R. Chauhan, and D.R. Roberts. 2006. A novel high-throughput screening system to evaluate the behavioral response of adult mosquitoes to chemicals. J. Am. Mosq. Contr. Assoc. 22 (in press)

Kongmee, M., A. Prabaripai, P. Akratanakul, M.J. Bangs, and T. Chareonviriyaphap. 2004. Behavioral responses of *Aedes aegypti* (Diptera: Culicidae) exposed to deltamethrin and possible implications for disease control. J. Med. Entomol. 41: 1055-1063.

Pell, J.K., M.A. Spinne, and K.J. Ward. 1989. Observations on the behavior of adult *Anopheles gambiae* encountering residual deposits of lambda-cyhalothrin compared with the other major classes, p. 18. In: Proceedings, 4th Annual Conference of the Society for Vector Ecology, European Region, Society for Vector Ecology, Santa Ana, CA.

Potikasikorn, J., T. Chareonviriyaphap, M.J. Bangs, and A. Prabaripai. 2005. Behavioral responses to DDT and pyrethroids between *Anopheles minimus* species A and C, malaria vectors in Thailand. Am. J. Trop. Med. Hyg. 73: 343-349.

Quinones, M.L. and M.F. Suarez. 1989. Irritability to DDT of natural populations of the primary malaria vectors in Colombia. J. Am. Mosq. Contr. Assoc. 5: 56-59.

Rachou, R.G., M.M. Lima, J.P. Duret and J.A. Kerr. 1966. Experiences with the excito-repellency test box—model ops. Rev. Bras. Malariol. Doencas. Trop. 18: 755-761.

Ree, H.I. and K.P. Loong. 1989. Irritability of *Anopheles farauti, Anopheles maculatus*, and *Culex quinquefasciatus* to permethrin. Japn. J. San. Zool. 40: 47-51.

Roberts, D.R., W.D. Alecrim, A.M. Tavares, and K.M. McNeil. 1984. Influence of physiological condition on the behavioral response of *Anopheles darlingi* to DDT. Mosq. News 44: 357-361.

Roberts, D.R., T. Chareonviriyaphap, H.H. Harlan, and P. Hshieh. 1997. Methods for testing and analyzing excito-repellency responses of malaria vectors to insecticides. J. Am. Mosq. Contr. Assoc. 13: 13-17.

Sungvornyothin, S, T. Chareonviriyaphap, A. Prabaripai, V. Trirakhupt, S. Ratanatham S, and M.J. Bangs. 2001. Effects of nutritional and physiological status on behavioral avoidance of *Anopheles minimus* (Diptera: Culicidae) to DDT, deltamethrin and lambdacyhalothrin. J. Vector Ecol. 26: 202-215.

Trophic behavior and biting activity of the two sibling species of the *Anopheles minimus* complex in western Thailand

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Received 6 April 2006; Accepted 23 May 2006

ABSTRACT: The trophic behavior and host preference of two sibling species, *Anopheles minimus* s.s. (= *An. minimus* species A) and species C, were observed during a two-year period at Pu Teuy Village, Sai Yok District, Kanchanaburi Province, western Thailand. *Anopheles minimus* s.s. and species C were more prevalent during the hot and wet periods of the year. Both species demonstrated exophagic and zoophilic activities. Feeding activity of *An. minimus* C was unique compared to *An. minimus* sensu lato from other localities in Thailand. Outdoor blood feeding by *An. minimus* C occurred throughout the night with one distinct feeding peak immediately after sunset (1800 h), whereas indoor feeding showed two small peaks at 2000 and 2400 h. The small number of *An. minimus* s.s collected during this study precluded a determination of peak activity patterns. A better understanding of mosquito behavior related to host and patterns of feeding activity will facilitate and improve the efficiency of vector control operations. ***Journal of Vector Ecology*** **31 (2): 252-261. 2006.**

Keyword Index: *Anopheles minimus*, species complex, sibling species, trophic behavior, bionomics, Thailand.

INTRODUCTION

Malaria remains the most significant vector-borne parasitic disease in the tropical and subtropical world. In Thailand, in spite of decades of well-organized malaria control activities, the burden of malaria still exists over much of the country. Malaria is particularly prevalent in the poorest of rural areas, especially along the national borders with eastern Myanmar, western Cambodia, and northern Malaysia (Chareonviriyaphap et al. 2000). These areas remain vulnerable to malaria transmission because of uncontrolled tribal population movement and political unrest. In many malaria endemic areas, *Anopheles minimus*, a mosquito common along the forest fringe zone, is an important malaria vector (Harrison 1980, Green et al. 1990, Chareonviriyaphap et al. 2000, Potikasikorn et al. 2005).

The *Anopheles minimus* complex, Theobald 1901, is currently composed of three sibling species in which two, *An. minimus s.s.* (= *An. minimus* species A) and *An. minimus* species C, are distributed in sympatry on the Asian mainland (Harbach 2004, Garros et al. 2006, Harbach et al. 2006). By definition, these species are difficult to accurately differentiate based on morphological characters (Rattanirithikul and Panthusiri 1994, Harrison 1980). *An. minimus* s.s. is the predominant species found throughout most of Thailand, whereas species C appears confined along the western Thai-Myanmar border, most notably in Kanchanaburi Province (Sucharit et al. 1988, Green et al. 1990, Garros et al. 2006). Several other putative species have been reported in Thailand, species D and n°157 (Sharpe et al. 1999), but information is lacking on the specific taxonomic status of these entities. Besides, it seems that species D is a chromosomal variant of *An. minimus* s.s. (Baimai, personal communication).

A better understanding of the biology and behavior of sibling species is critically important to help identify their respective role in disease transmission. Such information helps to define vector capacity, relative risk for disease transmission, and assists in the design of appropriate vector prevention and control strategies. Despite the existence in the literature of wing characteristics that could separate *An. minimus* s.s. from species C, recent rigorous studies have shown that morphological identification of the two sibling species of the Minimus Complex is not reliable and can lead to nearly 40% of misidentifications (Sungvornyothin et al. 2006, Jaichapor et al. 2005). Isoenzymes have served as the gold standard to separate the two sympatric species of the complex (Green 1990), however, this technique requires fresh or frozen specimens, and the complete destruction of the specimen makes impossible further studies such as sporozoite detection. More recently, molecular assays based on Polymerase Chain Reaction (PCR) were developed to identify *An. minimus* s.s. and species C, as well as the closely related sympatric species (Sharpe et al. 1999, Van Bortel et al. 1999, Kengne et al. 2001, Phuc et al. 2003, Garros et al. 2004a, b). The two Allele-Specific (AS)-PCR

assays were developed (Phuc et al. 2003, Garros et al. 2004b) for distinguishing through an easy, one-shot PCR, *An. minimus* s.s, species C, and three sympatric species, *An. aconitus, An. varuna,* and *An. pampanai.*

Recent studies on behavioral differences between *An. minimus* s.s. and C in northern Vietnam have shown that in sympatry, zoophilic behavior was pronounced for both species but species C was more exophagic and exophilic than *An. minimus* s.s. (Van Bortel et al. 1999, Trung et al. 2005). In non-sympatric situations, a wide range of behavior was observed for *An. minimus* s.s., leading to the conclusion that this species may exhibit high behavioral heterogeneities. In Thailand, *An. minimus* s.s. and C occur in sympatry in limited areas but few investigations have been conducted on each sibling species regarding feeding activity, resting behaviors, host preference (degree of anthropophily), and other bionomical factors that may influence their vector capacities. Rwegoshora et al. (2002) reported biting activity of *An. minimus* s.s. and species C in relation to seasonal climatic variations during the year and demonstrated greater outdoor feeding activity of species C. However, their study was based only on morphological identification of species with the high probability of misidentifications, and biting activity was not observed throughout the entire night (dusk to dawn). Recently, night-biting activity of *An. minimus* s.l. was also reported from Kanchanaburi Province, but these observations did not distinguish between species A and C (Chareonviriyaphap at al. 2003). Therefore, the aim of this work was to describe by using a molecular identification assay, the trophic behavior, biting activity, and seasonal abundance of the two sibling species of the Minimus Complex in western Thailand over a two-year period.

MATERIALS AND METHODS

Study area

The study was conducted in Pu Teuy, a village located in Sai Yok District, Kanchanaburi Province, western Thailand (14° 17'N, 99° 1'E). The rural site is located in mountainous terrain mostly surrounded by intact forest (Figure 1). The main water body near the collection site is a narrow, slow running stream, bordered with native vegetation (Chareonviriyaphap et al. 2003). This stream represents the main larval habitat for *An. minimus* s.l. (Kengluecha et al. 2005). Indoor residual spraying (IRS) using DDT had occurred routinely in this area for approximately 60 years (1940-2000), however, DDT was replaced by deltamethrin, a synthetic pyrethroid, in 2000. IRS was discontinued in the area during the course of our investigation.

Mosquito collections

Adult female mosquitoes were collected during three consecutive nights each month for two years, from February 2004 to January 2006. Three collection methods, indoor human-landing (HLI), outdoor human-landing (HLO), and cattle-bait collections (CBC), were used. The indoor/outdoor human-landing collectors were divided into two teams of four persons each. The first team worked from 1800 to 2400 h followed by the second team beginning at midnight to 0600 h. Human-landing collections occurred for 45 min each hour. Cattle bait collections was conducted by two collectors for 15 min each hour. Additional details on human landing collection methods are available in previous work (Chareonviriyaphap et al. 2003). Collected mosquitoes were retained in plastic cups labeled by hour and site of collection and covered with netting and cotton soaked with a 10% sugar solution placed at the top of the netting. Mosquitoes were returned to the laboratory for morphological identification the following morning. Hourly ambient outdoor temperatures and relative humidity were recorded at site. Rainfall data was obtained from the local Sai Yok District meteorological station located approximately 5 km from the study site.

Morphological and molecular species identification

Mosquito species were identified using the morphological keys of Peyton and Scanlon (1966) and Rattanarithikul and Panthusiri (1994). Following morphological identification, molecular identifications were performed using the multiplex AS-PCR assay of Garros et al. (2004b). Genomic DNA was extracted from

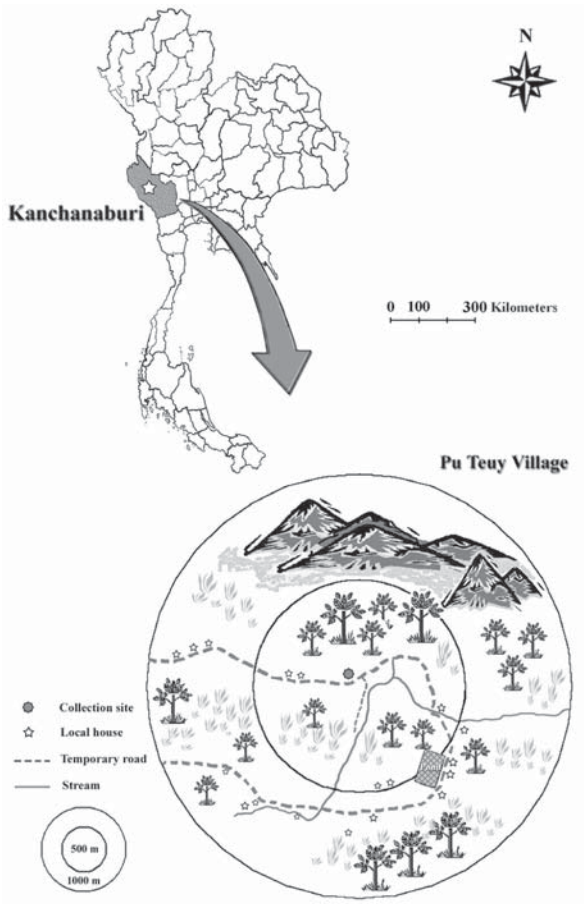


Figure 1. Study site of Pu Teuy Village, Kanchanaburi Province, west Thailand.

Table 1. Monthly frequency of *Anopheles* mosquitoes at Pu Teuy, Sai Yok District, Kanchanaburi Province, for two years (February 2004-January 2006).

Month	*An. minimus* s.s. No.	%	*An. minimus* C No.	%	*An. dirus* s.l. No.	%	*An. maculatus* s.s. No.	%	Total
Year 1									
Feb	46	11.9	335	86.6	1	0.3	5	1.3	387
Mar	15	6.9	192	88.6	0	0.0	9	4.2	216
Apr	7	12.3	46	80.7	3	5.3	1	1.8	57
May	21	6.0	215	66.4	47	13.4	50	14.2	333
Jun	28	4.3	256	39.0	139	21.2	233	35.5	656
Jul	21	4.5	150	32.0	42	9.0	256	54.6	469
Aug	8	6.4	63	50.4	38	30.4	16	12.8	125
Sep	4	3.9	39	38.2	47	46.1	12	11.8	102
Oct	3	3.8	61	77.2	8	10.1	7	8.9	79
Nov	7	4.1	132	78.1	1	0.6	29	17.2	169
Dec	15	6.8	195	88.2	0	0.0	11	5.0	221
Jan	9	4.1	206	94.9	0	0.0	2	0.9	217
Year 2									
Feb	16	6.9	214	93.1	0	0.0	0	0.0	230
Mar	20	3.7	513	95.6	0	0.0	4	0.7	537
Apr	20	3.7	444	78.2	0	0.0	2	0.1	466
May	13	5.7	178	83.5	5	2.3	18	8.5	214
Jun	19	3.2	380	65.2	63	10.7	123	20.9	585
Jul	3	2.3	45	34.4	47	35.8	36	27.5	131
Aug	3	0.7	97	25.4	79	19.5	221	54.4	400
Sep	6	4.8	44	35.5	74	59.7	0	0.0	124
Oct	13	5.3	156	64.8	73	29.9	0	0.0	242
Nov	22	7.1	263	92.6	1	0.3	0	0.0	286
Dec	9	4.5	218	95.5	0	0.0	0	0.0	227
Jan	13	5.7	216	94.3	0	0.0	0	0.0	229
Total	341	5.6	4658	76.7	668	11.0	1035	6.7	6702
Frequency per complex									
	Total 4999		74.6%		10.0%		15.4%		

Table 2. Total of monthly captures from three collection methods of *Anopheles minimus* species A and C.

	Indoor			Outdoor			Cattle bait		
Month*	*An. minimus* s.s	Species C	% Species C	*An. minimus* s.s	Species C	% Species C	*An. minimus* s.s	Species C	% Species C
Year 1									
Feb	1	3	75.0	6	76	92.7	39	256	86.8
Mar	0	12	100.0	3	28	90.3	12	152	92.7
Apr	2	8	80.0	0	5	100.0	5	33	86.8
May	2	21	91.3	6	26	81.3	13	168	92.8
Jun	0	9	100.0	3	29	90.6	25	218	89.7
Jul	0	10	100.0	2	15	88.2	19	125	86.8
Aug	2	4	66.7	0	10	100.0	6	49	89.1
Sep	0	8	100.0	0	4	100.0	4	27	87.1
Oct	0	0	0.0	3	14	82.4	0	47	100.0
Nov	0	2	100.0	1	19	95.0	6	111	94.9
Dec	0	4	100.0	4	67	94.4	11	124	91.8
Jan	0	1	100.0	3	31	91.2	6	174	96.7
Year 2									
Feb	0	0	0.0	0	20	100.0	16	194	92.4
Mar	0	2	100.0	4	76	95.0	16	435	96.4
Apr	0	1	100.0	1	17	94.4	19	426	95.7
May	1	2	66.7	0	15	100.0	12	161	93.1
Jun	1	27	96.4	4	67	94.4	14	186	93.0
Jul	0	2	100.0	0	4	100.0	3	39	92.9
Aug	0	9	100.0	0	38	100.0	3	50	94.3
Sep	1	0	0.0	0	1	100.0	5	43	89.6
Oct	0	4	100.0	3	24	88.9	10	128	92.8
Nov	0	1	100.0	8	51	86.4	14	211	93.8
Dec	0	3	100.0	1	45	97.8	8	170	95.5
Jan	0	1	100.0	2	34	94.4	11	181	94.3
Total	10	134	93.1	54	716	93.0	277	3808	93.2

* Three seasons defined as dry (Dec.-Feb.), hot (March-May), and wet (June-Nov.).

whole single adult mosquitoes according to procedures of Collins et al. (1987). The AS-PCR was conducted including the specific primers of *An. minimus* s.s. and species C, as well as the ones specific to the closely related species *An. aconitus*, *An. pampanai,* and *An. varuna* (Figure 2). In a volume of 25 µL template, PCR amplification conditions were as follows: 2.5 µL of 10x reaction buffer (Qiagen, Hilden, GR), 200 µM of each dNTP, 0.16 nmol of each primer, 0.5 units of *Taq* polymerase (Qiagen), and 2 µL of DNA template diluted 20 times. PCR cycles included one cycle at 94°C for 2 min, followed by 40 cycles at 94°C for 30 sec, 45°C for 30 sec, and 72°C for 40 sec each, followed by an final extension step at 72°C for 5 min. The PCR products were subjected to electrophoresis on a 3% agarose gel at 100 V for 30 min and stained with ethidium bromide (Figure 2)**.**

Data analysis

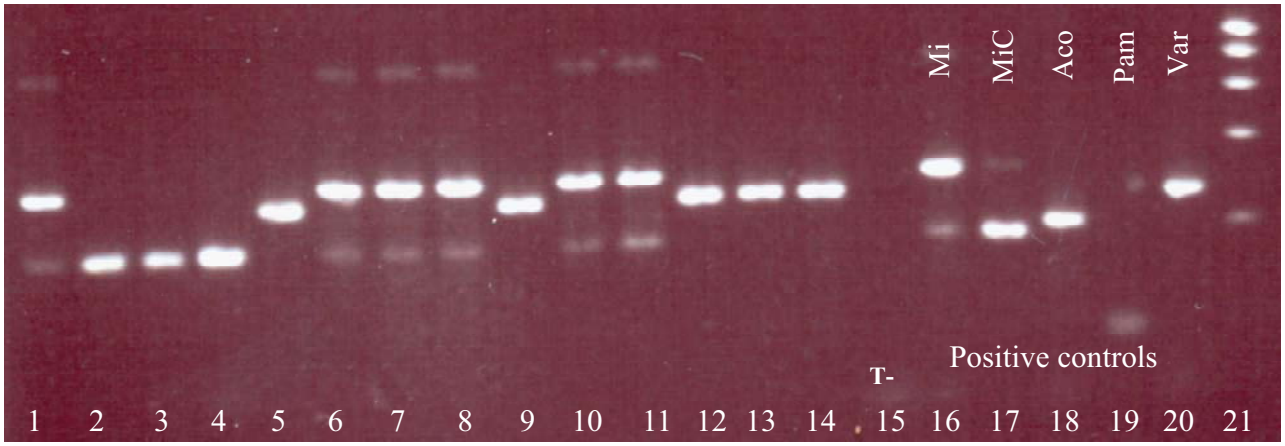
Seasonal differences based on average ambient temperature and precipitation and landing activity over hourly intervals during the evening (1800-0600) were selected for analysis in human/cattle landing collections. Seasons were classified as "dry" (December to February), "hot" (March to May), and "wet" (June to November). Time intervals were divided into early evening (1800-2100 h), late evening (2100-2400 h), pre-dawn (2400-0300 h), and dawn (0300-0600 h). Feeding habits and host preferences of each *An. minimus* species were classified as human indoor, human outdoor or cattle bait (outdoor). Nocturnal feeding cycles were tabulated by averaging the number of mosquitoes landing per human per night for indoor and outdoor collections and by averaging the number of mosquitoes captured per bovid per night. Comparisons of landing data were analyzed by a three–way analysis of variance (ANOVA), with year as the blocked factor. Differences among collection groups were determined by the Duncan multiple range test. All data were analyzed using SAS program package (SAS Release 6.10, SAS Institute, Cary, NC).

RESULTS

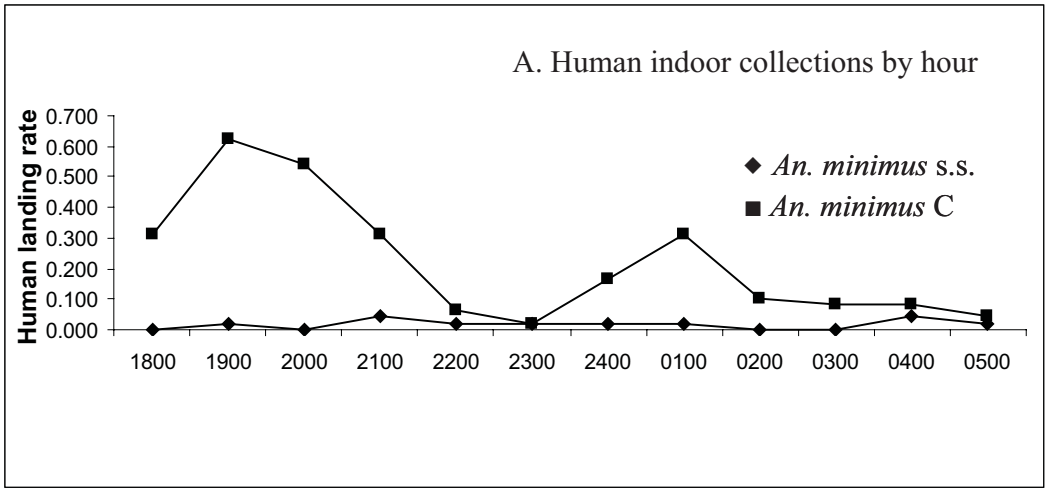
Observations on adult anopheline diversity, captured from February 2004 to January 2006 at Pu Teuy Village (Figure 1), are presented in Table 1. A total of 6,702 anophelines was collected during the 24 months of study. Members of three different anopheline vector complexes were collected throughout the year with the majority being *An. minimus* s.l. (74.6%). *Anopheles maculatus* s.s. and *Anopheles dirus* s.l., both important malaria vectors in Thailand, were collected in smaller proportions, representing respectively only 15.4% and 10% of the total collected anopheline fauna (Table 1). These two species complexes were found to be more abundant during the wet season, especially from June to September (Table 1). Two members of each complex were identified, such as *An. sawadwongporni* and *An. notanandai* for the Maculatus Complex, and *An. dirus* (former species A) and *An. baimaii* (former species D) for the Dirus Complex. In addition, limited numbers of *An. barbirostris, An. varuna, An. philippinensis, An. karwari, An. vagus, An. nivipes,* and *An. jamesii* were also collected (data not shown).

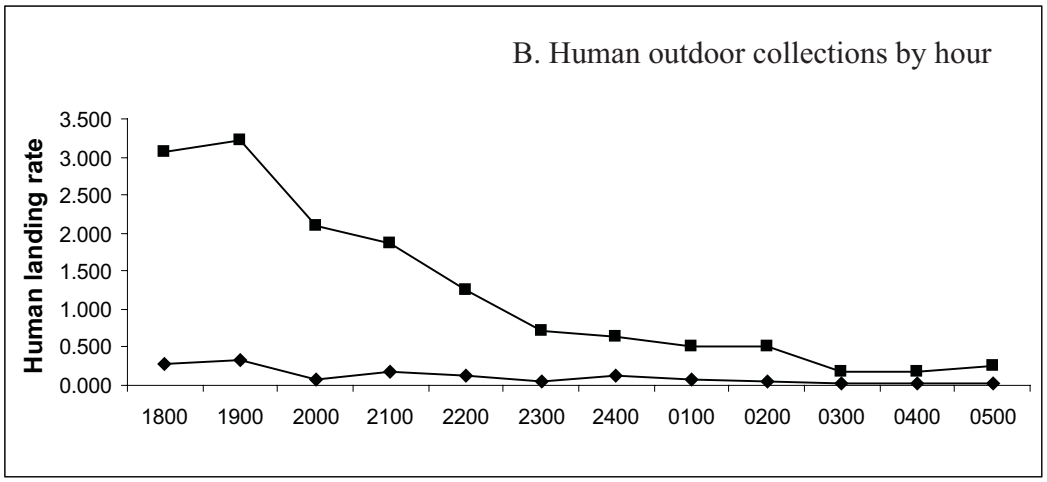
Molecular methods identified the two sibling species of the Minimus Complex, *An. minimus* s.s. and species C (Figure 2), with a much higher proportion (93.2%) of species C all over the two-year period. Table 2 provides the monthly distribution of *Anopheles minimus* s.l. collected by the three collection methods during the two-year period. A total of 4,999 adult females of *An. minimus* s.l. was tested, in which 4,658 (93.2%) were species C and 341 (6.8%) *An. minimus* s.s. A peak of seasonal abundance from April to July was particularly marked for *An. minimus* C during both years and also present for *An. minimus* s.s. but in much reduced proportions (Figure 4). Another smaller peak

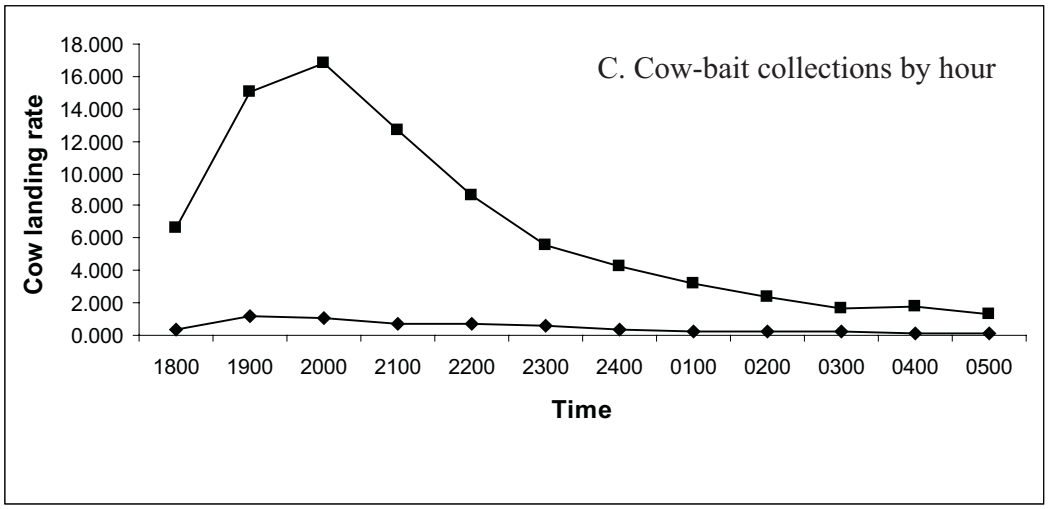
Figure 2. Multiplex Allele-Specific PCR assay. Lanes 1, 5, 9, 12-14: *An. varuna*; lanes 2-4: *An. minimus* C; lanes 6-8, 10, 11: *An. minimus* s.s.; lane 15: T-: negative control; lanes 16-20: Mi: *An. minimus* s.s.; MiC: *An. minimus* C; Aco: *An. aconitus*; Pam: *An. pampanai*; Var: *An. varuna*; lane 21: 200 bp molecular ladder.



Figures 3A-C. Evening blood feeding outdoor and indoor frequencies and host preference of *Anopheles minimus* s.s. and species C.







of abundance occurred for both species from October to December (Figure 4). For *An. minimus* species C, 81.8% (3,808) were captured on cattle, 15.4% (716) by outdoor human landing collection, and 2.9% (134) by indoor human landing collection (Table 2). Of a total of 341 specimens of *An. minimus* s.s. (species A), 81.2% (277) were captured on cattle, 15.8% (54) by outdoor human landing collection, and 2.9% (10) by indoor human landing collection (Table 2). Interestingly, the frequencies per collection method for each species were nearly identical. Overall, both species were more attracted to cattle than to humans and, in the latter case, more outdoors than indoors, regardless of the season.

Landing rates by hour and method for *An. minimus* s.s. and species C are illustrated in Figures 3a-c. *Anopheles minimus* C exceeded *An. minimus* s.s. in numbers for all collection hours, except for occasional early morning periods when both species were found in low densities. The indoor biting activity of *An. minimus* C presented two peaks, the largest peak around 1900 h and a smaller one at 0100 h (Figure 3a). The outdoor human landing activity for species C was elevated at the beginning of the capture (1800 h), immediately before dusk, reaching a peak around 1900 h, followed by a drastic decline in activity onwards throughout the evening (Figure 3b). Similarly, outdoor cattle bait catches showed one prominent peak for *An. minimus* C in the first quarter of the evening (1900-2100 h) followed by a decline throughout the night (Figure 3c). Because of the low numbers of *An. minimus* s.s. encountered, both indoor and outdoor activity peaks were difficult to discern and subject to greater bias (Figures 3a and b).

Total mosquitoes landing per hour were used in a three-way analysis of variance, with seasons (dry, hot, and wet), collection methods (indoors, outdoors, and cattle-baited) and time intervals (early evening, late evening, pre-dawn, and dawn) as discriminating factors. Species C varied statistically in mean number landing per hour among the three collection methods used ($F = 8.95$; df = 1, 11, $P = 0.0007$). The mean number captured on cattle was significantly greater than that of other collection methods ($P < 0.05$). Significant differences in mean number captured were observed between human outdoor and indoor collections ($P < 0.05$). Seasonal differences influenced mean number of captured mosquitoes, regardless of method ($F = 15.23$; df = 2, 11 $P < 0.0001$). Hourly means were significantly higher in the hot season than in either the wet or dry periods of the year ($P < 0.05$). A significant difference in mean number captured by time period was seen ($F = 12.98$; df= 1,11, $P = 0.0007$), with early evening (1800-2100 h) activity predominant ($P < 0.05$).

Data from all collection methods were pooled to determine the interaction between environmental factors and mosquito abundance. Species C biting activity was not correlated with increases in total rainfall and humidity ($r^2 = 0.29$, $P > 0.05$). Also, correlation between average minimum and maximum temperatures and feeding activity was not observed ($r^2 = 0.34$, $P > 0.05$).

DISCUSSION

Two sibling species of the Minimus Complex occur in Thailand, *An. minimus* s.s. and species C, which are known for their sympatry in Kanchanaburi Province. These two sibling species are impossible to accurately distinguish based on immature or adult morphological characters, which has complicated interpretation of previous findings based only on morphological identification (Kengluecha et al. 2005). Mosquitoes reported in this study were subjected to a multiplex AS-PCR, thus providing accurate species identification and describing with reliability the trophic behavior, seasonal abundance, and biting activity of *An. minimus* s.s. and species C in the village of Pu Tuey in Kanchanaburi Province.

Anopheles minimus C represented 93.2% of the *An. minimus* s.l. collected during the two-year period, which is consistent with previous observations in the same locality based on morphological identifications only. *Anopheles minimus* C was found to comprise 73-95% of the *An. minimus* s.l. captured in Pu Teuy (Green et al. 1990, Sucharit et al. 1988), and Rwegoshora et al. (2002) reported a species ratio of approximately 3:1 in favor of species C. Why this particular environment favors a significantly higher frequency of species C in the area is unknown but is likely related to local environmental or climatic factors that lend a competitive advantage to species C. Demographic changes resulting in increased deforestation and urbanization are often cited as contributors to changes in species distribution. However, our study site has remained in a natural environment, thus maintaining the same species composition over time. In the past, *An. minimus* s.l. populations have been reduced significantly in peninsular and southern Thailand and are also considered rare in the central plains of the country (Nutsathapana et al. 1986). Regular indoor residual spraying (IRS) for malaria control has been cited as a way to greatly reduce populations (Nutsathapana et al. 1986). This was also observed in the Terai and Himalayan foothills of Nepal where *An. minimus* s.l. was once considered the primary vector of hyperendemic malaria until DDT residual spraying reportedly eliminated the species completely from the area (Haworth 1988). Garros et al. (2005) also reported drastic and rapid changes in *An. minimus* s.l. species composition in central Vietnam following the introduction of permethrin-treated bednets, producing a significant reduction of *An. minimus* A along with the sudden increase of species C. In Thailand, *An. minimus* s.l. remains abundant in many foothill and forest fringe areas of the country, possibly the result of incomplete IRS coverage or inherent biological/behavioral differences (lower indoor resting and feeding behavior) in adult mosquitoes compared to other areas (Chareonviriyaphap et al. 2000, 2003, Potikasikorn et al. 2005). In general, there have been fewer environmental changes in foothill and forested areas that serve as stable habitats for *An. minimus* populations regardless of degree of IRS coverage. Unfortunately, the paucity of information on larval ecology of different members in the Minimus complex confounds

analysis and does not provide plausible explanations for species spatial distribution (Rattanarithikul et al. 1995, Kengluecha et al. 2005). Despite an intensive effort of larval habitat survey in Kanchanaburi Province, including Sai Yok District, Kengluecha et al. (2005) were unable to identify key environmental factors associated with *An. minimus* s.s. or species C. Their results implied that species distribution may be more associated with location of habitat rather than habitat type.

Pu Teuy village is considered nearly malaria-free, and only a few cases are documented each year. Our findings indicated that feeding habits of both species present a clear zoophilic behavior as they mainly feed on cattle located outside of living structures. In general, such feeding behavior, zoophily and exophagy, is considered less conducive to efficient and stable malaria transmission. Because *An. minimus* s.l., especially species C, was the predominant anopheline in Pu Teuy village during the two-year study, the low levels of malaria transmission in this area are likely the result of poor vectorial capacity, in particular because of the strong zoophilic tendency of both species. Actually, *An. minimus* s.s. is considered a relatively more efficient malaria vector than species C based on observed differences in host feeding behaviors (Green et al. 1990, Van Bortel et al. 1999, Trung et al. 2004). However, this study confirms that *An. minimus* s.s. and species C exhibited behavioral heterogeneities and are opportunist mosquitoes. In any case, the vectorial status of *An. minimus* C remains uncertain and the bionomics of this species requires further investigation. A low anthropophilic index and a strong tendency towards exophagy is in agreement with most studies on feeding behavior of *An. minimus* s.l. in Thailand (Ismail et al. 1978, Harrison 1980, Suthas et al. 1986, Rwegoshora et al. 2002, Chareonviriyaphap et al. 2003).

In Thailand, biting activity of *An. minimus* s.l. has been studied but never at the specific status. Harbach et al. (1987) observed a single biting peak between 2100-2200 h, whereas Ratanatham et al. (1988) reported two peaks, one in the early evening (1900-2200 h) and another before dawn (0500-0600 h). Rattanarithikul et al. (1996) found two prolonged feeding periods, the first wave occurring from 1800 to 2300 h, followed by a second wave from midnight until the pre-dawn hours. Our results of indoor human collections also showed two peaks for species C, similar to previous studies. In a sympatric area of northern Vietnam, the relative risk of being bitten before 2200 h was higher for species C compared to *An. minimus* s.s., whose peak feeding activity occurred after 2200 h (Trung et al. 2005). The limited number of *An. minimus* s.s. collected there did not allow an estimation of the feeding activity pattern.

Our study took advantage of PCR technology to identify the species of the Minimus Complex and thus describe individual biting cycles and blood-feeding activities. This information on the behavior of vector populations is crucial to explain the different levels of malaria risk based on the species in an area, which is essential for defining the most appropriate vector control strategies. A distinct biting pattern for species C was observed demonstrating a pronounced outdoor activity peak beginning around 1800 h until 1900 h, followed by a steady decline in landing numbers thereafter. Indoor activity was nearly 6-fold less than outdoor human landing counts, showing two modest peaks compared to outdoor populations, the largest at 1900-2000 h and a second, smaller peak around midnight-0100 h. Timing of indoor counts can be explained by an early evening delay in mosquito entry into dwellings followed by varying periods of pre-feed resting behavior before attacking a host (Roberts et al. 2000). Although we witnessed similar behavioral patterns with *An. minimus* s.s., the low numbers of specimens captured in Pu Teuy village precluded any definitive statistical descriptions about this member of the complex.

Acknowledgments

We thank the Thailand Research Fund (TRF) and Kasetsart University Research and Development Institute (KURDI) for financial support. Special thanks are extended to the students of the Department of Entomology, Kasetsart University who participated in mosquito collections.

REFERENCES CITED

Chareonviriyaphap, T., M.J. Bangs, and S. Ratanatham. 2000. Status of malaria in Thailand. Southeast Asian J. Trop. Med. Pub. Hlth. 31: 225-237.

Chareonviriyaphap, T., A. Prabaripai, M.J. Bangs, and B. Aum-Aung. 2003. Seasonal abundance and blood feeding activity of *Anopheles minimus* Theobald (Diptera: Culicidae) in Thailand. J. Med. Entomol. 40: 876-881.

Collins, F.H., M.A. Mendez, M.O. Rasmussen, P.C. Mehaffey, N.J. Besansky and V. Finnerty. 1987. A ribosomal RNA gene probe differentiates member species of the *Anopheles gambiae* complex. Am. J. Trop. Med. Hyg. 37: 37-41.

Garros, C., L.L. Koekemoer, L. Kamau, T.S. Awolola, W. Van Bortel, M. Coetzee, M. Coosemans, and S. Manguin. 2004a. Restriction fragment length polymorphism method for the identification of major African and Asian malaria vectors within the *Anopheles funestus* and *An. minimus* groups. Am. J. Trop. Med. Hyg. 70: 260-265.

Garros, C., L.L. Koekemoer, M, Coetzee, M. Coosemans, and S. Manguin. 2004b. A single multiplex assay to identify major malaria vectors within the African *Anopheles funestus* and the Oriental *Anopheles minimus* groups. Am. J. Trop. Med. Hyg. 70: 583-590.

Garros, C., R.P. Marchand, N.T. Quang, N.S. Hai, and S. Manguin. 2005. First record of *Anopheles minimus* C and significant reduction of *An. minimus* A in Central Vietnam. J. Am. Mosq. Contr. Assoc. 21: 139-143.

Garros, C., W. Van Bortel, H.D. Trung, M. Coosemans, and S. Manguin. 2006. Review of the Minimus Complex

of *Anopheles*, main malaria vector in Southeast Asia: from taxonomic issues to vector control strategies. Trop. Med. Int. Hlth. 11: 102-114.

Green, C.A., R.F. Gass, L.E. Munstermann, and V. Baimai. 1990. Population genetic evidence for two species in *Anopheles minimus* in Thailand. Med. Vet. Entomol. 4: 25-34.

Harbach, R.E. 2004. The classification of genus *Anopheles* (Diptera: Culicidae): a working hypothesis of phylogenetic relationships. Bull. Entomol. Res. 94: 537-553.

Harbach, R.E., J.B. Gingrich, and L.W. Pang. 1987. Some ecological observations on malaria transmission in a remote village in northwestern Thailand. J. Am. Mosq. Control Assoc. 3: 296-301.

Harbach, R.E., E. Parkin, B. Chen, and R.K. Butlin. 2006. *Anopheles* (*Cellia*) *minimus* Theobald (Diptera: Culicidae): neotype designation , characterization, and systematics. Proc. Entomol. Soc. Wash. 108: 198-209.

Harrison, B.A. 1980. The *Myzomyia* Series of *Anopheles* (*Cellia*) in Thailand, with emphasis on intra-interspecific variations (Diptera: Culicidae). Medical entomology studies-XIII. Contr. Am. Entomol. Inst. 17: 1-195.

Haworth, J. 1988. The global distribution of malaria and the present control effort. In: W.H. Wernsdorfer and I. McGregor (eds.). *Malaria: principles and practice of malariology*, Vol. 2, pp. 1379-1420. Churchill Livingstone, London.

Ismail, I.A.H., S. Phinichpongse, and P. Boonrasri. 1978. Responses of *Anopheles minimus* to DDT residual spraying in a cleared forested foothill area in central Thailand. Acta Trop. 35: 69-82.

Jaichapor, B., A. Kengluecha, P. Rongnoparut, L.M. Rueda, and R. Sithiprasasna. 2005. Morphological variations of *Anopheles minimus* A in Tak province, Thailand. Southeast Asian J. Trop. Med. Hyg. 36: 609-615.

Kengluecha, A., P. Rongnoparut, S. Boonsuepsakul, R. Sithiprasasna, P. Rodpradit and V. Baimai. 2005. Geographical distribution of *Anopheles minimus* species A and C in western Thailand. J. Vector Ecol. 30: 225-230.

Kengne, P., H. D. Trung, V. Baimai, M. Coosemans, and S. Manguin. 2001. A multiplex PCR-based method derived from Random Amplified Polymorphic DNA (RAPD) markers for the identification of species of the *Anopheles minimus* group in Southeast Asia. Insect Mol. Biol. 10: 427-435.

Nutsathapana, S., P. Sawasdiwongphorn, V. Chiprarop, and J.R. Cullen. 1986. The behavior of *Anopheles minimus* Theobald (Diptera: Culicidae) subjected to differing levels of DDT selection pressure in northern Thailand. Bull. Entomol. Res. 76: 303-312.

Peyton, E.L. and J.E. Scanlon. 1966. *Illustrated key to the female Anopheles mosquitoes of Thailand. Bangkok*. U.S. Army Medical Component, Southeast Asia Treaty Organization.

Phuc, H.K., A.J. Ball, L. Son, N.V. Hanh, N.D. Tu, N.G. Lien, A.Verardi, and H. Townson. 2003. Multiplex PCR assay for malaria vector *Anopheles minimus* and four related species in the Myzomyia Series from Southeast Asia. Med. Vet. Entomol. 17: 423-428.

Potikasikorn, J., T. Chareonviriyaphap, M.J. Bangs, and A. Prabaripai. 2005. Behavioral responses to DDT and pyrethroids between *Anopheles minimus* species A and C, malaria vector in Thailand. Am. J. Trop. Med. Hyg. 73: 343-349.

Ratanatham, S., E.S. Upathem, C. Prasittisuk, W. Rojanasunan, N. Theerasilp, A.Tremongkol, and V. Viyanant. 1988. Bionomics of *Anopheles minimus* and its role in malaria transmission in Thailand. Southeast Asian J. Trop. Med. Publ. Hlth. 19: 283-289.

Rattanarithikul, R. and P. Panthusiri. 1994. Illustrated keys to the medically important mosquitoes of Thailand. Southeast Asian J. Trop. Med. Publ. Hlth. 25 (Suppl): 1-66.

Rattanarithikul, R., C.A. Green, S. Panyim, C. Noigamol, S. Chanaimongkol, and P. Mahapibul. 1995. Larval habitats of malaria vectors and other *Anopheles* mosquitoes around a transmission focus in northwestern Thailand. J. Am. Mosq. Contr. Assoc. 11: 428-433.

Rattanarithikul, R., E. Konishi, and K.L. Linthicum. 1996. Detection of *Plasmodium falciparum* circumsporozoite antigen in anopheline mosquitoes collected in southern Thailand. Am. J. Trop. Med. Hyg. 54: 114-121.

Roberts, D.R., W.D. Alecrim, P. Hshieh, J. Grieco, M.J. Bangs, R.G. Andre, and T. Chareonviriyaphap. 2000. A probability model of vector behavior: effects of DDT repellency, irritability, and toxicity in malaria control. J. Vector Ecol. 25: 48-61.

Rwegoshora, T.R., R.G. Sharpe, K.L. Baisley, and P. Kittayapong. 2002. Biting behavior and seasonal variation in the abundance of *Anopheles minimus* species A and C. Southeast Asian J. Trop. Med. Publ. Hlth. 33: 694-701.

Sharpe, R.G., M.M. Hims, R.E. Harbach, and R.K. Butlin. 1999. Two PCR based methods for identification of species of the *Anopheles minimus* group: allele specific amplification and single strand conformation polymorphism. Med. Vet. Entomol. 13: 265-273.

Sucharit, S., N. Komalamisra, S. Leemingsawat, C. Apiwathnasorn, and S. Thongrungkiat. 1988. Population genetic studies on the *Anopheles minimus* complex in Thailand. Southeast Asian J. Trop. Med. Publ. Hlth. 19: 717-723.

Sungvornyothin, S., C. Garros, T. Chareonviryaphap, S. Manguin. 2006. How reliable is the humeral pale spot for identification of cryptic species of the Minimus Complex? J. Am. Mosq. Contr. Assoc. 22: 185-191.

Suthas, N., P. Sawasdiwongphorn, U. Chitprarop, and J.R. Cullen. 1986. The behavior of *Anopheles minimus* Theobald (Diptera: Culicidae) subjected to different levels of DDT selection pressure in northern Thailand. Bull. Entomol. Res. 76: 303-312.

Trung, H.D., W. Van Bortel, W. Sochantha, T. Keokenchanh, N.T. Quang, L.D. Cong, and M. Coosemans. 2004.

Malaria transmission and major malaria vectors in different geographical areas of southeast Asia. Trop. Med. Int. Hlth. 9: 230-237.

Trung, H.D., W. Van Bortel, T. Sochanta, K. Keokenchan, O. Briet, and M. Coosemans. 2005. Behavioural heterogeneity of *Anopheles* species in ecologically different localities in Southeast Asia: A challenge for vector control. Trop. Med. Int. Hlth. 10: 251-262.

Van Bortel, W., H.D. Trung, N.D. Manh, P. Roelants, P. Verlé, and M. Coosemans. 1999. Identification of two species within the *Anopheles minimus* complex in northern Vietnam and their behavioural divergences. Trop. Med. Int. Hlth. 4: 257-265.

Journal of the American Mosquito Control Association, 22(4):689–698, 2006

INSECTICIDE-INDUCED BEHAVIORAL RESPONSES IN TWO POPULATIONS OF *ANOPHELES MACULATUS* AND *ANOPHELES SAWADWONGPORNI*, MALARIA VECTORS IN THAILAND

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ABSTRACT. Behavioral responses of 2 wild-caught populations of *Anopheles maculatus* (Theobald) and *Anopheles sawadwongporni* Rattanarithikul and Green to operational field doses of DDT (2 g/m^2) and permethrin (0.5 g/m^2) were characterized using an excito-repellency test system. Both test populations, collected from animal quarters at Ban Pu Teuy, Sai Yok District, Kanchanaburi Province, western Thailand, were found completely susceptible to DDT and permethrin. Specimens from 2 test populations quickly escaped from direct contact with treated surfaces from 2 insecticides compared with paired controls. Noncontact repellency response to DDT was significantly pronounced in *An. sawadwongporni* ($P < 0.05$) and comparatively weak in *An. maculatus*, but it was statistically greater than individually paired controls ($P < 0.05$). We conclude that contact irritancy is a major behavioral response of both field populations when exposed directly to DDT and permethrin, whereas noncontact repellency to DDT also produced a significant escape response in *An. sawadwongporni*.

KEY WORDS Behavioral avoidance, irritancy, repellency, *Anopheles maculatus*, *Anopheles sawadwongporni*, DDT, permethrin, Thailand

INTRODUCTION

Members of the *Anopheles (Cellia) maculatus* complex are important vectors of malaria throughout the Oriental Region, including Thailand, Indonesia, Malaysia, and the Philippines (Reid 1968). This complex contains at least 8 closely related species and is differentiated based on variability in morphological, behavioral, and genetic characters (Green et al. 1985, Rattanarithikul and Green 1986, Chiang et al. 1991, Kittiyapong et al. 1993, Bangs et al. 2002). In Thailand, 6 species have been reported, including *An. maculatus* s.s. Theobald, *Anopheles sawadwongporni* Rattanarithikul and Green, *Anopheles dravidicus* Christophers, *Anopheles notanandai* Rattanarithikul and Green, *Anopheles willmori* (James), and *Anopheles psuedowillmori* (Theobald) (Green et al. 1985, Rattanarithikul and Green 1986, Rattanarithikul and Harbach 1990, Kittayapong et al. 1990, Green et al. 1992). Three species have been incriminated as important vectors of malaria in Southeast Asia, including *An. maculatus* s.s. (Reid 1968), *An. willmori* (Pradham et al. 1970), and *An. pseudowillmori* (Green et al. 1991). *Anopheles sawadwongporni* is a common species often found in high density throughout Thailand, especially along the border provinces with Myanmar and Malaysia (Disease Control Department 2005), and this species has been shown to be an important vector of *Plasmodium falciparum* in the country (Rattanarithikul et al. 1996).

For decades, DDT was routinely used for malaria control as an indoor residual spray (IRS) in Thailand. DDT use was halted for all public health use in 2001 after a progressive phaseout period beginning in 1995 (Chareonviriyaphap et al. 2000). The reasons for DDT removal from the malaria control inventory were politically and operationally based. A combination of cost and the gradual increase of poor community compliance to IRS in some areas all contributed to the chemical being removed permanently from organized malaria control (Chareonviriyaphap et al. 1999). Interestingly, the development of DDT resistance by vector mosquitoes was not documented in Thailand or provided as a reason for terminating its use. DDT was gradually replaced by two potent pyrethroids, deltamethrin and permethrin (Chareonviriyaphap et al. 2000). From the beginning, deltamethrin has been used primarily for IRS and permethrin applied for treatment of netting material used in bed-nets and curtains (Chareonviriyaphap et al. 2004, Disease Control Department 2005).

Although DDT was withdrawn, it was done without good understanding or appreciation of the impact its loss would have on vector populations in terms of behavioral avoidance and malaria transmission reduction. Behavioral responses of mosquitoes to insecticides influence vectorial capacity of vectors by altering or disrupting normal behavioral activity (Sparks et al. 1989, Klowden 1996, Costantini et al. 1999).

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Additional research is needed to verify avoidance responses of different vector populations to insecticides in the field (Chareonviriyaphap et al. 1997, van Bortel et al. 2004, Potikasikorn et al. 2005).

Insecticide avoidance includes contact irritancy and noncontact repellency (Roberts et al. 1997a). Irritant responses result from physical contact with chemically treated surfaces, whereas repellency is an avoidance response devoid from having made actual contact with insecticides (Lockwood et al. 1984, Chareonviriyaphap et al. 1997, Roberts et al. 1997a) Previous studies characterized avoidance behavior to deltamethrin with laboratory and field populations of anophelines, including *An. maculatus* and *An. sawadwongporni* (Chareonviriyaphap et al. 2004).

The evaluation of DDT remains important as a standard comparison against other residuals. The assessment of excito-repellent (ER) properties of DDT retains merit in that this once commonly used and successful compound might offer options for limited reintroduction in malaria areas of Thailand that have been refractory to current control methods. Permethrin, in contrast, has been primarily used in bed-nets in malarial areas. Although this compound is used on a relatively small scale, its impact on *An. maculatus* and *An. sawadwongporni* remains unknown. Described here is the first study of both insecticides by using an ER test to quantify behavioral responses between wild-caught populations of *An. maculatus* and *An. sawadwongporni* exposed to recommended "operational" doses of DDT and permethrin.

MATERIALS AND METHODS

Test populations

Female mosquitoes were obtained from Ban Pu Teuy, Sai Yok District, Kanchanaburi Province, western Thailand (91°110'N, 97°113'W) from March to November 2005. Both populations were 100% susceptible to diagnostic concentrations of DDT (4%) and permethrin (0.75%) by using standard WHO bioassay contact tests (Chareonviriyaphap, unpublished data).

Mosquito collections

Mosquitoes were collected off cattle by using mouth aspirators during 15 min each hour from 1800 to 2400 h. Mosquitoes were held in a plastic cup covered with wet cotton until identification the following morning. *Anopheles maculatus* and *An. sawadwongporni* were separated in holding cages until testing. Behavioral assays were performed on unfed mosquitoes within 48 h of capture.

Insecticide-treated papers

Analytical grade insecticide was impregnated on papers at single standard operational field concentrations of 2 g/m^2 of DDT and 0.5 g/m^2 of permethrin prepared according to WHO protocol (WHO 1998). All papers were treated at the rate of 2.75 ml of the insecticide solution per 180 cm^2.

Excito-repellency tests

The rationale and analysis for ER test data have been described in detail elsewhere (Roberts et al. 1997a). A full test consisted of a pair of treatment chambers and a pair of control chambers. One treatment chamber permitted tarsal contact with insecticide-treated papers. The second treatment chamber included the inner chamber, so mosquitoes could not make contact with insecticide-treated papers. Treatment chambers were lined with test papers that were impregnated with insecticide and an oil-based carrier (risella oil). Control chambers were lined with papers that were impregnated with carrier alone. For brevity, tests with or without the inner chambers, for either treatment or control papers, are referred to as contact trials (no inner chamber) or noncontact trial (with inner chamber).

For a complete test, 25 mosquitoes were introduced into each of 4 chambers by using a mouth aspirator. After the mosquitoes were put in the chamber, the outer rear door was closed and secured. A receiving cage, a 6 × 6 × 6-cm paper box, was connected to the exit window for collecting escaping specimens. At the start of the test, a 3-min rest period was used to permit mosquitoes to adjust to test chamber conditions (Busvine 1964, Chareonviriyaphap et al. 1997). After 3 min, the escape funnel was opened to initiate the observation period. Numbers of mosquitoes escaping from the exposure chamber into the receiving cage were recorded at 1-min intervals (Chareonviriyaphap et al. 2002).

Tests compared 2 wild-caught populations in contact versus noncontact exposures by using DDT and permethrin. Mosquitoes were deprived of blood and sugar approximately 12 h before tests. Ambient temperatures and relative humidity were recorded during the ER assays and 24-h postexposure holding periods. All tests were performed in the field during daylight, and each test series was replicated at least 3 times. After each test period, the numbers of dead or knockdown specimens were recorded separately from each exposure chamber, escape holding cage, and paired control chambers. Live escaped specimens and those remaining inside the treatment and control chamber were collected and held separately in small holding containers topped with cotton soaked with 10% sugar solution until 24-h mortalities were recorded.

Table 1. Percentage of escape response and mortality of *Anopheles maculatus* (MAC) and *Anopheles sawadwongporni* (SAW) to DDT and permethrin in contact and noncontact trials.

							% mortality			
			Treatment		Control		Treatment		Control	
Test	Chemical	Species	No. tested	% esc[3]	No. tested	% esc	Esc	Not esc[4]	Esc	Not esc
Contact	DDT[1]	MAC	93	38	97	5	9	31	0	1
		SAW	93	37	97	7	18	20	0	1
	PER[2]	MAC	96	76	99	17	7	22	0	0
		SAW	94	64	89	15	8	6	0	1
Noncontact	DDT	MAC	95	14	94	8	9	0	0	1
		SAW	94	37	94	12	3	3	0	0
	PER	MAC	92	27	88	14	20	13	0	0
		SAW	91	26	91	18	4	0	0	0

[1] DDT 2 g/m^2.
[2] PER, permethrin 0.5 g/m^2.
[3] Esc, escaped.
[4] Not esc, not escaped.

Data analysis

A Kaplan–Meier survival analysis method was used to analyze and interpret the behavioral response data (Roberts et al. 1997a). Survival analysis was used to estimate the probability of escape time (ET) and compare differences in mosquito response between the 2 field populations and two insecticides (Kleinbaum 1995). Mosquitoes that escaped were treated as "deaths," and those remaining in the test chamber were treated as "survivals" (Chareonviriyaphap et al. 1997). The ET_{25} and ET_{50} values, the time in minutes for 25% and 50% of the test population to escape, respectively, were estimated from the data. The log-rank method was used to compare patterns of escape response between treatment groups (Mantel and Haenzel 1959). Statistical software (STATA®, Stata Corporation, College Station, TX) was used in the analysis. Statistical significance for all tests was set at $P < 0.05$.

RESULTS

This study compared behavioral responses of wild-caught populations of *An. maculatus* and *An. sawadwongporni* females exposed to a single standard field dose of DDT (2.0 g/m^2) and permethrin (0.5 g/m^2). Both contact irritancy and noncontact repellency were observed in both test populations. Percentages of mortalities of escape and nonescape mosquitoes from control and treated chambers were recorded (Table 1). Contact trials with permethrin produced patterns and rates of escape significantly stronger in both mosquito populations than those exposed to DDT ($P < 0.05$). After 30-min exposure, the escape response to DDT was similar between populations (38% for *An. maculatus* and 37% for *An. sawadwongporni*). In contrast, far stronger escape responses were observed in both populations against permethrin (76% *An. maculatus* and 64% *An. sawadwongporni*). In noncontact trials, repellency to DDT was more pronounced in *An. sawadwongporni* (37%) than in *An. maculatus* (14%). Permethrin produced greater repellency responses than DDT in *An. maculatus* (27%) and *An. sawadwongporni* (26%). Overall, fewer females escaped from treated chambers without direct insecticidal contact, but the repellency response was still statistically different from that of the paired controls (Table 1).

Percentages of mortalities of recovered specimens after a 24-h holding period after contact and noncontact assays are presented in Table 1. In contact trials, percentages of mortalities of escaped specimens were low, ranging from 8% to 18% for DDT and from 7% to 8% for permethrin. Percentage of mortality was moderately higher for those mosquitoes that remained in the test chamber, ranging between 20% and 31% for DDT and between 6% and 22% for permethrin. In noncontact trials, the percentages of mortalities of escaped and nonescape specimens were low (0–9%), except for escapees (20%) and nonescapees (13%) of *An. maculatus* exposed to permethrin (Table 1).

The escape patterns from insecticide-treated chambers expressed in 1-min intervals to statistically derive the ET_{25} and ET_{50} values were calculated. In contact trials, the ET_{25} values for *An. maculatus* and *An. sawadwongporni* were 8 and 3 min for DDT and 4.5 and 5 min for permethrin, respectively. The ET_{50} values for *An. maculatus* and *An. sawadwongporni* were 9 and 13 min for permethrin, respectively. The ET_{50} values for DDT against both species could not be estimated because of insufficient numbers of mosquitoes escaping. In noncontact trials for *An. sawadwongporni*, the ET_{25} value for DDT and permethrin was 12 and 26.5 min, respectively.

Table 2. Comparison of escape responses between *Anopheles maculatus* and *Anopheles sawadwongporni* to insecticide in contact and noncontact trials.

Chemicals	Contact exposure	Noncontact exposure
DDT[1]	0.9729	0.0001[2]
PER[3]	0.1100[2]	0.9066

[1] DDT 2 g/m².
[2] Log rank tests with statistically significant ($P < 0.05$) differences in patterns of escape.
[3] PER, permethrin 0.5 g/m².

Statistical comparisons of escape responses between any 2 populations in contact and noncontact trials to DDT and permethrin are presented in Table 2. Both mosquito populations showed very similar responses. In contact and noncontact trials with DDT and permethrin, no statistical differences in escape responses were observed in all designed pairings ($P > 0.1$), except when comparing *An. maculatus* and *An. sawadwongporni* against DDT in noncontact assays ($P < 0.0001$).

All escape patterns were significantly different comparing contact with noncontact, contact with paired controls, and noncontact with paired controls in exposures to DDT and permethrin against both populations, with only 2 exceptions: DDT contact versus noncontact pairs with *An. sawadwongporni* ($P = 0.83$), and DDT noncontact versus controls in *An. maculatus* ($P = 0.48$) (Table 3).

Figures 1–3 illustrate the proportions of mosquitoes remaining in treatment and control chambers during 30-min time under different test conditions and chemical exposure. These proportions were used to develop escape rate patterns of probability for escaping from exposure chambers in contact and noncontact assays (Fig. 1), contact and paired control (Fig. 2), and noncontact and paired control designs (Fig. 3). There were significant differences in irritancy seen in all contact versus control pairings (Fig. 1). Strong repellency to DDT was observed in *An. sawadwongporni*, with significantly less escape observed with *An. maculatus* (Fig. 3). In DDT noncontact trials, no differences in escape patterns of *An. maculatus* were observed between treatment and control ($P > 0.05$) (Table 3 and Fig 3), indicating very low repellency despite a greater percentage of test specimens escaping from the treated chamber than controls (Table 1).

DISCUSSION

Excito-repellency to insecticides by mosquitoes has been recognized for >60 years, yet it remains a relatively poorly studied and underappreciated phenomena. One reason for the apparent inattention has been the lack of a single accepted method for the quantitative assessment of behavioral responses. Some of the impassiveness toward ER also has stemmed from past difficulties gathering data, analyzing data, and interpreting the concepts and significance of the findings (Roberts et al. 1984, Evans 1993, Chareonviriyaphap et al. 1997, Rutledge et al. 1999, Sungvornyothin et al. 2001, Potikasikorn et al. 2005). It has been nearly a decade since Roberts et al. (1997a) developed an ER test box that allows for the direct observation of the 2 primary types of intrinsic behavioral avoidance, irritancy and repellency (Chareonviriyaphap et al. 1997). Admittedly, the prototype device was rather cumbersome and required considerable time to attach the test papers on the inner walls of the chambers. Based on the same conceptual design, Chareonviriyaphap et al. (2002) provided an improved version of the ER test chamber that has proven successful on numerous occasions for accurate evaluation behavioral responses of various mosquito vectors in Thailand (Sungvornyothin et al. 2001; Kongmee et al. 2004; Chareonviriyaphap et al. 2001, 2004; Potikasikorn et al. 2005). Recently, improvements have been made with a modular, high-throughput assay system for rapid mass screening of test compounds and behavioral responses of adult mosquitoes (Grieco et al. 2005). This novel, laboratory-based, system can effectively differentiate the 3 primary attributes of many insecticides: contact irritancy, spatial repellency, and toxicity.

Table 3. Comparison of escape responses between contact versus noncontact, contact versus control, and noncontact versus control for *Anopheles maculatus* (MAC) and *Anopheles sawadwongporni* (SAW) by insecticides.

Species	Chemical	Treatment pairs		
		Control vs. contact	Contact vs. noncontact	Noncontact vs.control
MAC				
	DDT[1]	0.0001	0.0001	0.4875[2]
	PER[3]	0.0001	0.0001	0.0001
SAW				
	DDT	0.0001	0.8296[2]	0.0001
	PER	0.0001	0.0001	0.0180

[1] DDT 2 g/m².
[2] Log rank tests showing no statistically significant ($P > 0.05$) differences in escape patterns.
[3] PER, permethrin 0.5 g/m².

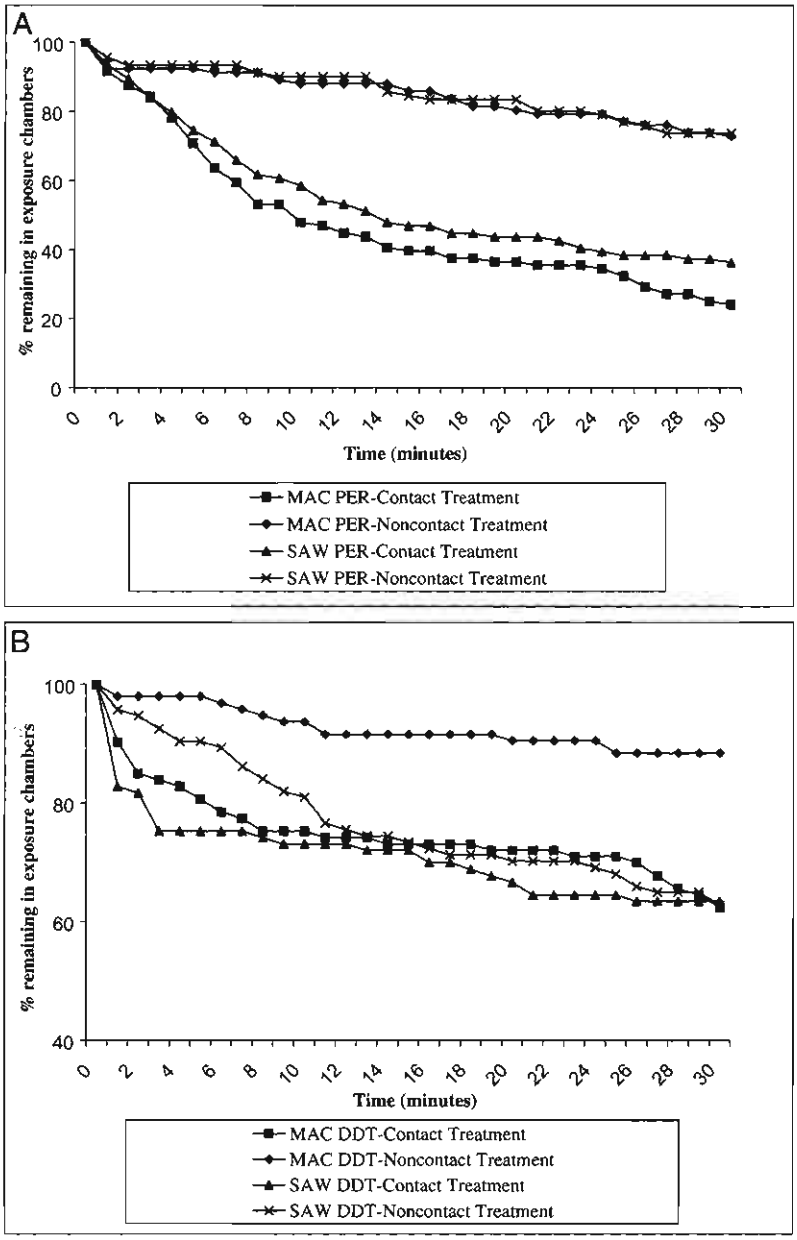

Fig. 1. Proportions of *Anopheles maculatus* and *Anopheles sawadwongporni* females remaining in paired contact and noncontact trials after exposure to either (A) 0.5 g/m² permethrin or (B) 2 g/m² DDT.

Excito-repellency has been investigated in several mosquito species in Thailand; however, no ER testing as yet has conducted on wild-caught populations of *An. maculatus* and *An. sawadwongporni*, both important vectors of malaria in Thailand, to DDT and permethrin. For decades, DDT was used in Thailand extensively for intradomicilary application, once or twice each year, to control malaria vectors (Prasittisuk 1985, Chareonviriyaphap et al. 1999, Potikasikorn et al. 2005). Despite the widespread success of DDT for malaria vector control in the past (Roberts et al. 1997b, 2000b, 2004), the impact of behavioral avoidance in terms actual malaria transmission reduction has remained controversial (Roberts et al. 2000a). The Government of

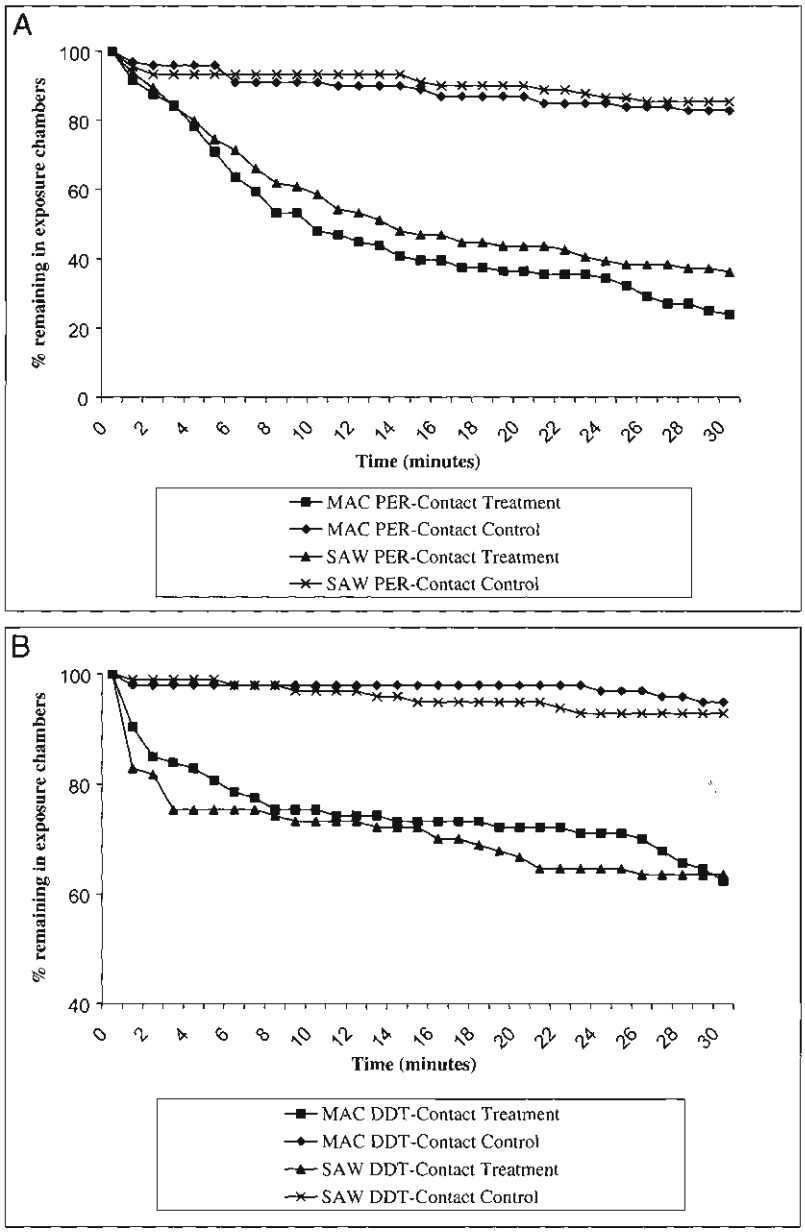

Fig. 2. Proportions of *Anopheles maculatus* and *Anopheles sawadwongporni* females remaining in paired contact versus control trials after exposure to either (A) 0.5 g/m^2 permethrin or (B) 2 g/m^2 DDT.

Thailand terminated use of DDT for malaria control in 2001, concentrating on use of pyrethroids for IRS and bed-net treatment. However, DDT remains effective and safe when applied to interior walls of homes, a conclusion drawn by worldwide consensus in December 2000 (UNEP 2000). The final acceptance of DDT by this international forum and the Stockholm Convention on Persistent Organic Pollutants for continued use in the benefit of public health is clear testament to its unique effectiveness to combat malaria and the realization that the relatively small amounts required for indoor spraying have very limited effect on the environment while sparing countless lives from malaria in endemic countries (UNDP 2001, Roberts et al. 2004).

Roberts et al. (2000a) examined the properties of DDT in malaria control and empirically