Inactivation of transmission capability of disease vector by entomopathogenic fungi

December 2016

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昆虫寄生性糸状菌による病原体ベクターの媒介能インアクティベーション

平成 28 年 12 月

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Chapter III

An entomopathogenic fungus strain of *Beauveria bassiana* inactivate disease transmission and reproductive capability against *Anopheles stephensi*

GENERAL INTRODUCTIONS

Hosts and their parasites interactions

Hosts and their parasites share a long history of association in the common habitats where they endure similar environmental conditions. In the long history, they are interacted each other and evolved. Among them, the types of associations between hosts and their parasites are divided into two groups: interaction in which parasite act as pathogen against insects, and interaction in which parasite form mutualistic associations with insects. In the former, it was though that hosts and their parasites have each strategy to obtain their fitness. Various studies reported that parasites change the specificity, virulence and behaviors against their hosts in their interactions (e.g. Bridge, 1997, Leal et al., 1997, St. Leger, 1992, Alizon et al., 2009 and Webster, 2007). And also in entomopathogens, several studies reported that fungal pathogen often alter or manipulate their host's behavior. Fungal pathogen, *Ophiocordyceps unilateralis sensu lato*, could control insect brains and manipulate their behavior to reach death locations that are optimal for spore dispersal (Andersen et al., 2009). Unlike normal behavior, infected ants walk alone and erratically climb to a certain height in the vegetation, and then bite leaf margins in rainforests and twigs in temperate woods and transition from wandering to biting takes place synchronously within 11:00–14:00 h possibly in association with a solar cue (Bekker et al., 2014 and Hughes et al., 2011). Infected pea aphids, *Acyrthosiphon pisum*,

were moved to the undersides of leaves by *Pandora neoaphidis* infection (Jensen et al., 2001), and *Lecanicillium longisporum* infection caused an increase in activity at the beginning of mycosis against green peach aphid, *Myzus persicae* (Roditakis et al, 2008). It is thought that these alterations or manipulations for the behavior were strategy of entomopathogenic fungi for diffuse fungal pathogens. On the other hand, some behavior alteration contributes as host defense mechanisms from the pathogen attack. In order to prevent fungal infection, infected hosts (e.g. grasshopper and flies) altered their thermoregulatory behavior and showed a behavioral fever response to fungal pathogens (Carruthers et al., 1992, Watson et al., 1993, Blanford et al., 1998 and Ouedraogo et al., 2004). These phenomena might be occurred by co-evolution between insect hosts and their fungal pathogens. In insects and pathogens co-evolution, whereas selection pressure of fungal pathogen is greater exploitation of the host, selection pressure of insect host is for greater overcoming the pathogen (Roy et al., 2005 and Baverstock et al., 2010). Regardless of their strategy and/or fitness, it was reported that fungal pathogens alter their hosts behaviors in several studies. *M. anisopliae* infection reduced blood feeding and fecundity against *An. gambiae* (Scholte et al., 2006), and *Beauveria bassiana* showed not only lethal effect but also sub-lethal effects such as reduction of response to the frequency of glucose and blood feeding against *Anopheles stephensi* (Blanford et al., 2011).

Probably, behavioral alterations by fungal pathogens might generally exist on each host-fungus pair in a nature. Although these interactions and mechanisms are interest points in evolutionary biology, understanding the fundamental behavioral processes that occur between insect hosts and fungal pathogens is also essential for insect pathology to exploit entomopathogenic fungi as biological control agents.

History of entomopathogenic fungi

Historically, entomopathogenic fungi especially *Beauveria* spp. had been recognized as disease against beneficial insects (e.g. silkworm and honey bee). Rehner (2005) reviewed that *Beauveria* spp. was discovered at a time when the existence of fungi and other microbes and their biological roles, particularly as agents of plant and animal diseases, were first being discovered. The Italian lawyer and scientist, Agostino Bassi (1773-1856) first found that the cause of the disease of silkworm in microorganism in 1834, and then the disease was formally name the muscardine fungus *Botrytis bassiana* (later named *Beauveria bassiana*) in honor of Bassi, facilitating scientific and technical communications about organism confirming the etiology of the disease. Additionally, scientific studies by Agostino Bassi demonstrating that *Beauveria* was the infectious disease agent that caused the white muscardine disease of silkworm were important

antecedents to the germ theory of disease, arguably one of the most significant theories in the history of science. In the study of Bassi's inoculation confirming the etiology of the disease, it was reported that this disease was not restricted to silkworm but also caused disease in other insect species by Audoin in 1837. After several decades, entomopathogenic fungi had begun to be recognized as beneficial agent for controlling agricultural pests. In second half of the nineteenth century, Metchnikoff in 1879 and Krassilstschik in 1888 first reported that mass-produced *Metarhizium anisopliae* was tested preparations for control of the wheat cockchafer, *Anisoplia austriaca*, and sugarbeet curculionid, *Cleonuspu punctiventris* (Gillespie, 1988). In brief, although investigation of *Beauveria* was instigated by the need to protect a beneficial domesticated insect, *Beauveria* is also an important natural pathogen of insects, its hosts including many economically important insect pests. As a result, a great deal of research on entomopathogenic fungi has been motivated, and then various species of entomopathogenic fungi such as *B. bassiana* and *M. anisopliae* are studied and used for the control of several agricultural insect pests. However, the discovery and use of chemical insecticides in the 1940s overshadowed the potential of entomopathogenic fungi. Thereafter, development of insecticide resistance was a serious problem around the world, and entomopathogenic fungi have been focused on agriculture again, as an alternative

approach to insect pest control.

Entomopathogenic fungi as biocontrol agent

Several species of entomopathogenic fungi were commercialized worldwide and used for very wide range insect families, e.g. Aleyrodidae (Ignoffo and Anderson, 1979), Chrysomelidae (Kreutz et al., 2004, Wraight et al., 2001 Feng et al., 1994, Ferron, 1981 and Ignoffo and Anderson, 1979), Curculionidae (Feng et al., 1994), Crambidae (Wraight et al., 2001), Scarabaeidae and Noctuidae (Copping et al., 2004), Alyeyrodidae, Thripidae, Tetranchidae and Aleyrodiae (Shternshis, 2004 and Alves et al., 2003), Tortricidae (Feng et al., 1994, Ferron, 1981 and Ignoffo and Anderson 1979), Muscidae (Kaufman et al., 2005), Crambidae (Hajek et al., 2001, Shah and Goettel, 1999), Miridae, Cicadellidae, Fulgoridae, Aleyrodidae, Aphididae, Pseudococcidae, Psyllidae, Thripidae and Tettigoniidae (Kabaluk and Gazdik, 2005, Wraight et al., 2001) , Castniidae (Alves et al., 2003), Cerambycidae (Hajek et al., 2001, 2006 and Wraight et al., 2001), Ericophyidae (Copping, 2004, Kumar and Singh, 2001, McCoy, 1978 and 1996), Culicidae (Scholte et al., 2004), Kalotermitidae, Rhinotermitedae and Termopsidae (Wraight and Carruthers, 1999 and Rath, 1995), Battellidae and Blattidae (Evan, 2003, Hajek et al., 2001 and Gunner et al., 1995), Cercopidae (Alves et al., 2003), and Pyrgomorphidae (Pettit and

Jenkins, 2005 and Bateman, 1997), reviewed by (Faria and Wraight, 2007). Moreover, they are also act as fungal endophyte and plant disease antagonists. Some fungal endophytes protect host plants against plant pathogens and herbivores (Arnold et al., 2003 and Arnold and Lewis, 2005), and many fungi traditionally known as insect pathogens have been isolated as endophytes, including *Beauveria* and *Isaria* spp. (Vega et al., 2008). Some entomopathogenic fungi such as *B. bassiana* and *Lecanicillium* spp. also are antagonistic to plant disease, e.g. powdery mildews, green mold and *Rhizoctonia solani* (Askary et al., 1998, Benhamou and Brodeur, 2001, Kim et al., 2008, Ownley et al., 2004 and 2008). Moreover, *Lecanicillim* spp. also have pathogenicity against plant parasitic nematodes (e.g. cyst nematode) (Shinya et al., 2008a, 2008b and 2008c). It seems likely that entomopathogenic fungi can be used in multiple roles in protecting plants from pests and diseases.

Entomopathogenic fungi have two groups, Entomophthorales as specialist and Hyphomycetes as generalist based on the spectrum of host, and they include more than 700 known fungal species from 100 genera (Hajek, 2004), most of the commercially produced fungi are species of *Beauveria*, *Metarhizium*, *Lecanicillium* and *Isaria* spp. that are relatively easy to mass produce (Vega et al, 2009). Hyphomycetes have numerous advantage for use as biological control agents. First, they are cosmopolitan fungi,

relatively easy to recognition, and frequent appearance in nature. Second, they have the extremely broad host range. Especially, *B. bassiana* is known to infect more than 700 species of insects (Goettel et al., 1990), and its wide variation in virulence toward different insect hosts. Third, they are an extremely tractable organism because it is easily isolated from insect cadavers or from soil by using simple media, antibiotics, and selective agents (Beilharz et al., 1982 and Chase et al., 1986), and by baiting soil with insects (Zimmermann, 1986). Additionally, they flourish in the laboratory on simple media (Goettel and Inglis, 1997) and can be conserved by storage in glycerol solutions at ultra-low temperatures or by freeze-drying (Humber, 2001). Hyphomycetes include about 20 genera of entomopathogenic fungi, e.g. *Beauveria* spp., *Metarhizium* spp., *Lecanicillium* spp., *Isaria* spp., *Aschersonia* spp., *Nomuraea* spp. and *Hirsutella* spp. which causes of muscardine disease of insects, and they are used for control of agricultural insect pests widely around the world. During the last four decades, 171 fungal biocontrol agents have been commercialized by over 80 companies worldwide. Among them, the 6 fungal biocontrol agents have been resisted and marketed in Japan (Faria and Wraight, 2007). Furthermore, entomopathogenic fungi have been used for not only the agricultural pests but also sanitary insect such as ticks, tsetse flies and assassin bugs (Kirkland et al., 2004, Maniania et al, 2013 and Vázquez-Martínez et al, 2014).

Vector borne diseases and insecticide resistance

Vector borne diseases are remaining as a major public health, and the pathogens are transmitted by the blood feeding of arthropod species, such as mosquitoes, ticks and assassin bugs. Vector mosquitoes are closely associated with mankind since time immemorial and play an important role in the transmission of various diseases such as malaria, Japanese encephalitis, filariasis, dengue and yellow fever. Especially, malaria is the most serious vector borne disease in the world (WHO, 2015). Malaria remains an important cause of illness and death in countries in which it is endemic. Malaria control is mainly conducted by vector control and prompt treatment with effective antimalarial agents. Chemical insecticides targeting to the vector have been one of the most successful strategies employed for malaria control (Hemingway and Ranson, 2000). Although DDT and pyrethroid insecticide have been mainly used for the vector control, the effectiveness and sustainability of insecticide-based interventions, such as indoor residual sprays (IRS) and insecticide-treated nets (ITNs), are being undermined by development of insecticide resistance (Hemingway and Ranson, 2000 and Hargreaves et al., 2000). It was reported that the evidence of accumulation of malaria vector resistance to commonly used insecticides in several malaria endemic countries worldwide, including Burkina Faso, Côte d'Ivoire, South Africa, Ghana, Equatorial Guinea, Angola, Gabon, Benin, Ethiopia

and Congo–Brazzaville (WHO, 2012). The development of insecticides resistance might jeopardize the current vector control efforts. Hence, knowledge of vector resistance is the basic elements to guide insecticide selection and use in the vector control programs (Van Bortel et al., 2008). Now establishment of alternative non-chemical approaches were required, and the World Health Organization (WHO) strongly recommends the simultaneous use of different vector control tools and this has formulated the basis for Integrated Vector Management (IVM) strategies (van den Berg et al., 2011).

Alternative approach for vector mosquito control

IVM is defined as a rational decision-making process for the optimal use of resources for vector control and includes five key elements: 1) evidence-based decision-making, 2) integrated approaches 3), collaboration within the health sector and with other sectors, 4) advocacy, social mobilization, and legislation, and 5) capacity-building. In integrated approach among them, integration of an alternative (non-chemical) and chemical vector control methods is required (Beier et al., 2008). In an alternative approach, biological control agents primarily involve the use of *Bacillus thuringiensis* (Fillinger and Lindsay, 2006), larvicidal fishes (Chandra et al., 2008), and aquatic fungi (Scholte et al., 2004) against mosquito larvae. Entomopathogenic fungi are also used for the vector mosquito

control. In control of mosquito larvae stage, entomopathogenic fungus *Aspergillus flavus* was reported having pathogenicity against mosquito species *Aedes fluviatilis* and *Culex quinquefasciatus* (Moraes et al., 2001), and *Aspergillus clavatus* have pathogenicity to *Ae. aegypti*, *An. gambiae* and *Culex quinquefasciatus* (Seye et al., 2009). Clark et al. (1968) reported that the conidia of *B. bassiana* are effective in killing mosquito larvae when applied as a conidial dust to the water surface of breeding sites. However, mosquitoes have a variety of habitation places depending on the growth stage. For instance, the larval habitats include not only hydrosphere of the large quantity of wetlands but also a little puddle such as an empty can, an old tire and the wheel track (Scholte et al., 2004). It was difficult to effectively control the larvae stage. On the other hand, in the cause of targeting to the adult stage, we could restrict control area (e.g. indoor house and shed for animals). Over the last decade, there has been increasing focus on the use of entomopathogenic fungi to control adult mosquitoes (Scholte et al., 2003 and Scholte et al., 2004). This approach uses fungal entomopathogens as novel active ingredients in biopesticides (Blanford et al., 2005). Unlike other biocontrol agents (such as bacteria, microsporidia and viruses), these fungi infect and kill insects without needing to be ingested. Tarsal contact alone is enough to kill the mosquito, which is a characteristic that is shared with insecticidal chemicals (Farenhorst et al., 2010). This characteristic is expected to be an

important factor for controlling adult mosquitoes. For instance, Scholte et al. (2005) developed a practical system for the delivery of the fungus *Metarhizium anisopliae* to adult *Anopheles gambiae* by using oil-based conidia-impregnated cotton sheets in the houses of rural villages in Tanzania and it could infect 23% of female *An. gambiae* resting on the cloths, shortening the average mosquito life span by 4 to 6 days compared with controls (Scholte et al., 2005). In the study of laboratory, 6 fungal isolates were shown to induce mosquito mortality of more than 80% within 14 days (Blanford et al., 2005). Furthermore, it was reported that the insecticide-resistant strains of *An. gambiae* were more highly susceptible to infection of *B. bassiana* and *M. anisopliae* than the insecticide-susceptible strain of *An. gambiae* (Farenhorst et al., 2010). This finding is important in considering the involvement of entomopathogenic fungi in IVM activities in situations where resistance to synthetic insecticides is evident.

The objects in this doctoral dissertation

Understanding of relationship between vector mosquitoes and entomopathogenic fungi is beneficial information for vector mosquito control. I have mainly three question between vector mosquitoes and entomopathogenic fungi. How do the mosquitoes and fungi interact each other on an ecological system and the natural environment? How do the

fungi invade in the mosquitoes, and then kill them? Do the fungi have some kind of effects excluding lethal effect against the mosquitoes? Although these elucidations are extremely interesting in ecology and evolutionary biology, these understanding will also become the powerful weapon for mosquito control. In previous studies for several decades, numerous researches of the mosquito control by entomopathogenic fungi have been conducted. However, there are only few studies that elucidated the relations between vector mosquitoes and entomopathogenic fungi. These new findings will be applied to establishment of efficient and practicable control of vector mosquitoes.

In this doctoral dissertation, I would describe novelty aspect for understanding the relationship between the vector mosquito and entomopathogenic fungi. In order to obtain the information between mosquitoes and entomopathogenic fungi in a natural state, entomopathogenic fungi were isolated from wild mosquitoes collected in Japan and Burkina Faso and the original fungal library which is specialized against vector mosquito control was established (Chapter I). And to screen useful candidates as microbial control agent (e.g. high virulence and infectivity), 69 fungal isolates isolated from the wild mosquitoes were applied to bioassay against *An. stephensi* (Chapter I). Subsequently, to understanding of infection dynamics between *An. stephensi* and *B. bassiana sensu lato* 60-2 (which showed highest virulence and infectivity in Chapter I) by tarsus inoculation

method, fungal adhesion parts to mosquito body and fungal invasion rate to each mosquito parts were evaluated. Additionally, fungal invasion rates to each mosquito parts were compared with mortality and infection level to detect lethal factor (Chapter II). Finally, to elucidate some kind of effects by entomopathogenic fungi infection against the mosquitoes, the host searching behaviors to each attractant, blood feeding and egg production were evaluated (Chapter III). These works might be able to elucidate the relationships between the mosquitoes and entomopathogenic fungi and also the total performance of entomopathogenic fungi as the vector control agent.

Chapter I

Evaluation of the pathogenicity and infectivity of entomopathogenic fungi, isolated from wild mosquitoes in Japan and Burkina Faso, against female adult *Anopheles stephensi* mosquitoes

I-1. Introduction

Entomopathogenic fungi, including *Beauveria* spp., *Metarhizium* spp., *Isaria* spp., and *Lecanicillium* spp., are already being produced commercially for use against a large number of agricultural insect pests worldwide (Faria and Wraight, 2007). *M. anisopliae* and *B. bassiana* are effective at killing both chemical insecticide-resistant and insecticide-susceptible adult mosquitoes (Howard et al., 2010). Currently, vector mosquito control using entomopathogenic fungi is under intense investigation. However, most published studies have used entomopathogenic fungi isolated from species other than those belonging to the mosquito family Culicidae. Therefore, information remains limited about the relationship between mosquitoes and entomopathogenic fungi in the natural state. Only a few entomopathogenic fungi isolated from wild mosquitoes have been listed in culture collection institutes worldwide. For instance, only 40 isolates in the United States Department of Agriculture–Agricultural Research Service Collection of Entomopathogenic Fungi, three isolates in the American Type Culture Collection, and four isolates in the Centraalbureau voor Schimmelcultures Fungal Biodiversity Centre are listed as entomopathogenic fungi isolated from mosquitoes. Furthermore, *B. bassiana* is not included among these entomopathogenic fungi. *Beauveria* is one of the most frequently isolated entomogenous fungal genera because of its cosmopolitan distribution

(Rehner et al., 2011). Although *Beauveria* has a very broad host range, the natural occurrence of *Beauveria* in or on mosquitoes has only been reported in a limited number of studies. For instance, *B. bassiana* was isolated from *Culex pipiens* larvae (Kal'vish and Kukharchuk, 1974), while *Beauveria tenella* was found to be a naturally occurring pathogen in field populations of *Aedes sierrensis* larvae (Pinnock et al., 1973). In addition, *Beauveria* sp. was isolated from wild adult mosquitoes of the species *Cx. tarsalis*, *Cx. pipiens*, and *An. albimanus* (Clark et al., 1968). Other fungal species also infect mosquito larvae in the natural state, including *Metarhizium* spp., *Paecilomyces* spp., and *Lecanicillium* sp. (Scholte et al., 2004). However, there is a paucity of previous reports about these species infecting adult mosquitoes. Thus, fundamental and multilateral understanding about the ecological state of the mosquito–entomopathogenic fungi relationship may make valuable contributions to the biological control of vector mosquitoes.

In this study, we aimed to understand the natural state of the relationship between mosquitoes and entomopathogenic fungi. Specifically, entomopathogenic fungi were isolated from wild adult mosquitoes collected in a temperate non-malaria endemic country (Japan) and from a malaria endemic country (Burkina Faso). These specimens were used to establish a specialized culture library of entomopathogenic fungi obtained

from mosquitoes. Furthermore, the virulence and infectivity of these fungal isolates against *An. stephensi* were evaluated. The results of this study are anticipated to help identify promising isolates as fungal biopesticides against malaria vector mosquitoes.

Materials and methods

I-2.1. Collection of adult mosquitoes in Japan

Adult mosquitoes were collected between Jun. 2009 and Sept. 2009 in Hokkaido, Japan, using sweeping techniques and human bait or the Center for Disease Control Light Trap (CDC-LT) with dry ice. Collected mosquitoes were killed by refrigeration at −30 °C to maintain the mycobiota located inside the body and on the external body surface of the insects in a viable condition until fungal isolation.

I-2.2. Collection of adult female *An. gambiae sensu lato* in Burkina Faso

Adult female *An. gambiae* were collected during the dry season (Nov. 2008 and 2009) in the villages of Goden and Koubri, Burkina Faso. Mosquitoes were collected from the inside of houses built of brick. A cloth was unfurled on the floor, and the house was sprayed with insecticide (KALTOX®: containing 0.27 % Allethrin, 0.2 % Tetramethrin,

0.17 % Permethrin, and 0.68 % Propoxur). After a few minutes, dead mosquitoes fell on the cloth. Only female *An. gambiae* were selected and packed in test tubes that were chilled until fungal isolation (~4 °C).

I-2.3. Fungal isolation and morphological identification

Frozen mosquitoes collected on the same date and from the same location were placed in the same plastic tube containing 1.0 ml sterile distilled water, and were homogenized using a pestle. The resulting suspensions, which contained from 1 to 52 individuals, were plated on selective medium for entomopathogenic fungi. The medium contained 10 g glucose, 10 g peptone, 15 g Oxygal, 60 mg Rose Bengal, 0.5 g chloramphenicol, 10 mg dodine, 0.25 g cycloheximide, 60 mg streptomycin, 60 mg penicillin G potassium, and 30 g agar l^{-1} sterile distilled water (Goettel and Inglis, 1997). A 0.2 ml volume of the suspension was plated on five separate plates. The plates were incubated at 24 °C in the dark for approximately 12 days. The fungal colonies that grew on the medium were then transferred to potato dextrose agar (PDA) plates. The fungal isolates detected from mosquitoes were identified using morphological and molecular-based techniques. All fungal isolates were stored in 25 % glycerol solution at −80 °C. Morphological identification to the genus level was conducted by the slide culture method under a light

microscope (Leica: DMI 3000B) at 200–400× magnification.

Furthermore, frozen mosquitoes from Japan were surface-sterilized by sequential washes in 70 % ethanol for 1 min, rinsed twice with sterile distilled water for 1 min, and allowed to surface dry under sterile conditions. The specimens were then transferred to separate plastic tubes containing 0.2 ml of sterile distilled water. Subsequently, each mosquito was homogenized in the tubes. The same methods described above were followed for isolation and identification. After the second rinse with sterile distilled water, the samples were plated on entomopathogenic fungi selective medium, and then fungal growth was checked to ensure that sterilization had been successful.

I-2.4. Fungal isolation from the puddle water of a Japanese mosquito breeding site

Puddle water was collected from a Japanese mosquito breeding site. A 0.2-ml volume of water was plated on entomopathogenic fungi selective medium. The same methods described above were followed for isolation and identification. This experiment was conducted with 10 replicates, and the fungal colonies on each plate were counted.

I-2.5. Molecular-based identification

For molecular-based identification, genomic DNA was extracted using the method

described by Saitoh et al. (2006). PCR primers used for the universal fungal amplification of the internal transcribed spacer (ITS) region were ITS 5 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990). The PCR mixture (20 µl) contained 0.1 U Taq DNA polymerase (Takara Bio Inc., Japan), 1× PCR buffer, 0.2 mM of each dNTP, 10 µM of each primer, and 1 µl of the template DNA. PCR was performed in a GeneAmp® PCR System 9700 (Applied Biosystems, Tokyo, Japan). Thermal conditions were as follows: denaturing at 94 °C for 2 min; 40 cycles at 94 °C for 30 s, 57 °C for 30 s, 72 °C for 1 min; and a final extension at 72 °C for 7 min. Then, the PCR products were purified using Nucleospin® Extract II (Takara Bio Inc., Japan), according to the manufacturer's instructions. Amplified PCR products were run on 2 % agarose gel, stained with ethidium bromide, and the amplicons were visualised under UV light. Then, the PCR products were sequenced using a 3730xl DNA Analyzer with a Big Dye Terminator v3.1 (Applied Biosystems, Tokyo, Japan). Species were identified by database searches using the BLAST sequence analysis tool (http://blast.ncbi.nlm.nih.gov/blast.cgi). The ITS sequence was compared using nucleotide BLAST (blastn). Species identification was determined from the lowest expected value of the BLAST output.

I-2.6. Mosquito rearing

The larvae of *An. stephensi* were reared for approximately 12 days in plastic cups (8 cm in diameter and 4.5 cm in height or 10.5 cm in diameter and 6 cm in height) that were filled with tap water. Subsequently, the larvae were transferred to plastic trays (20.5 × 26.5 × 4.5 cm). The larvae were fed daily with Hikari Economy® fish food. The pupae were collected daily and transferred to mesh cages (27 × 27 × 27 cm). Adults that emerged were fed a 10 % (w/v) sucrose/water solution *ad libitum*. At all developmental stages, mosquitoes were kept in an incubator (27 ± 0.4 °C and a 12-h light:dark [L:D] photoperiod). For the bioassay, 4–6-days-old female adult mosquitoes were transferred from the adult cage to a glass bottle (2.7 cm in diameter and 12 cm in height) using a mouth aspirator.

I-2.7. Fungal preparation

The fungal isolates from wild mosquitoes were cultured on a 90 mm PDA plate at 24 °C in the dark for 10–15 days. The spore concentration was adjusted to 1.3×10^7 conidia ml^{-1} in 0.05 % Tween 20 solution. One milliliter of this suspension was pipetted evenly over a 90 mm diam filter paper, resulting in conidial densities of 2.0×10^{10} conidia/m^2. This filter paper was then placed in the lid of a 90-mm-diameter Petri dish. After

inoculation, the viability of the conidia was assessed by placing the conidial suspension on a 1.5 % water agar (WA) plate and incubating it at 24 °C in the dark for 48 h. Subsequently, the plate was observed under a light microscope at 200× magnification to determine the proportion of germinated conidia. Three hundred conidia were evaluated from each replicate. The germination rate of all fungal isolates used in this study was more than 93.6 %.

I-2.8. Preliminary bioassay for evaluating the virulence and infectivity of entomopathogenic fungi against *An. stephensi*

A bioassay was conducted to evaluate the virulence and infectivity of 36 fungal isolates from Japan without surface sterilization, 10 fungal isolates from Burkina Faso without surface sterilization, and 23 fungal isolates from surface-sterilized mosquitoes from Japan against laboratory-reared adult female *An. stephensi* (Table 1-1). Thirty mosquitoes were transferred from the adult cage to an acrylic bottle and were anesthetized with carbon dioxide gas. The mosquitoes were transferred to a Petri dish that was prepared as described in the previous section and were placed in direct contact with the fungal inoculum on the filter paper. After recovering from the anesthesia, the mosquitoes were allowed to walk on the inoculum for 30 min. After exposure, the mosquitoes were

transferred to bottom-meshed assay tubes (8.5 cm in diameter and 9.5 cm in height), which were sealed with nylon socks with the toe part cut off (Farenhorst and Knols, 2010). The mosquitoes were kept at 27 ± 0.4 °C, 72 ± 1 % relative humidity and a 12 h light:dark photoperiod. They were fed with 10 % (w/v) sucrose/water solution *ad libitum*. Mortality was monitored daily, until all mosquitoes were dead. Cadavers were then frozen at −30 °C. This experiment was conducted using two separate replicates.

Thirty frozen mosquitoes from the virulence assay were immersed in 70 % ethanol for 1 min, rinsed twice in sterilized water for 1 min, and incubated on a 1.5 % WA (water agar) plate at 24 °C in the dark. After 10 days, the mosquitoes were assessed for hyphal growth using an inverted microscope. The sporulation rate of the 30 mosquitoes was also assessed. This experiment was conducted after each virulence assay.

I-2.9. Bioassay for selected isolates that showed higher virulence and/or infectivity against female adult *An. stephensi*

After the bioassay for virulence and/or infectivity, the isolates that showed higher virulence and/or infectivity were selected to conduct three additional replicate experiments using the same technique as that used to estimate the survival rate of mosquitoes. Eleven isolates from Japan without surface sterilization, three fungal isolates

from Burkina Faso without surface sterilization, and 12 isolates from surface-sterilized mosquitoes from Japan were selected. In total, 26 isolates were used in the bioassay (Table 1-2).

I-2.10. Statistical analysis

For all treatments, median survival time (MST) and survival rates were determined using Kaplan–Meier survival analyses, with significant differences between treatments and controls estimated using a log rank test. Abbott's formula was used to correct for natural mortality in all bioassays. The mortality rate of adult female mosquitoes between treatment and control groups was analyzed using ANOVA. When the F test was significant at $P < 0.01$, the treatment means were compared using Tukey's honestly significant difference test. Arcsine transformation was used for all percentage datasets.

I-3. Results

I-3.1. Japan: Isolation of entomopathogenic fungi from wild mosquitoes without surface sterilization

A total of 1026 individuals of *Aedes* spp. and 12 individuals of *Culex* spp. from Japan

were used to isolate entomopathogenic fungi. In total, 5366 fungal colonies were isolated on selective medium, including *Cladosporium* spp., *Aspergillus* spp., *Geomyces* spp., *Microascus* spp., *Pseudocercospora* spp., *Trametes* spp., *Peniophora* spp., *Acremonium* spp., *Penicillium* spp., *Stachybotrys* spp., *Neonectria* spp., and *Fusarium* spp. Of these colonies, 237 isolates of entomopathogenic fungi were identified as 81 isolates of *B. bassiana s.l.*, four isolates of *B. brongniartii*, 29 isolates of *Isaria farinosa*, one isolate of *Isaria* sp., 14 isolates of *Lecanicillium araneicola*, 95 isolates of *Lecanicillium* spp., four isolates of *Paecilomyces carneus*, eight isolates of *Simplicillium lamellicola*, and one isolate of *S. lanosoniveum* (Table 1-3).

I-3.2. Burkina Faso: Isolation of entomopathogenic fungi from wild mosquitoes without surface sterilization

A total of 1685 female adults of *An. gambiae* were collected in Burkina Faso and used for fungal isolation. In total, 13080 fungal colonies were detected on the selective medium for entomopathogenic fungi, including *Cladosporium* spp., *Aspergillus* spp., and *Pseudocercospora* spp. Of these colonies, 94 isolates of entomopathogenic fungi were identified as three isolates of *B. bassiana s.l.*, one isolate of *I. farinosa*, 77 isolates of *L. araneicola*, and 13 isolates of *S. lanosoniveum* (Table 1-4).

I-3.3. Japan: Isolation of entomopathogenic fungi from surface-sterilized wild mosquitoes

A total of 447 individuals of *Aedes* spp. and 5 individuals of *Culex* spp. from Japan were used for the isolation of entomopathogenic fungi. In total, 731 fungal colonies were detected on the selective medium for entomopathogenic fungi. Of these colonies, 64 isolates of entomopathogenic fungi were identified as 40 *B. bassiana s.l.* isolates, one isolate of *I. farinosa*, one isolate of *I. fumosorosea*, and 22 isolates of *Lecanicillium* spp. (Table 1-5). The latent infection rate among active mosquitoes (which were captured live using a hand net) was 4.7 %. Moreover, two co-infection cases were observed, in which both *B. bassiana s.l.* and *Lecanicillium* sp. were isolated from one individual.

I-3.4. Fungal isolation from the puddle water of a Japanese mosquito breeding site

B. bassiana s.l., *Lecanicillium* spp., and *Isaria* spp. were obtained from the puddle water at a Japanese mosquito breeding site at a density of 1.5 CFU ml^{-1}, 8.0 CFU ml^{-1}, and 3.0 CFU ml^{-1}, respectively.

I-3.5. Preliminary bioassay to evaluate the virulence and infectivity of entomopathogenic fungi against female adult *An. stephensi*

In the preliminary bioassay for virulence of 36 fungal isolates from Japan without surface sterilization, nine isolates of *B. bassiana s.l.*, two isolates of *B. brongniartii*, six isolates of *Lecanicillium* spp., four isolates of *I. farinosa*, and two isolates of *P. carneus* showed significantly higher mortality against *An. stephensi* compared with the control ($P < 0.001$). Of these isolates, *B. bassiana s.l.* isolates 26-8, 31-37, 58-11, 59-45, and 60-2 and *Lecanicillium* sp. isolates 27-15 and 63-15 killed more than 90 % of *An. stephensi* within 14 days of exposure. *B. bassiana s.l.* isolate 60-2 showed the highest virulence of all isolates. The MST ranged from 6.2 to 18.3 days for adults treated with fungi and was 19.5 days for the control (Table 1-1).

In the preliminary bioassay of 10 fungal isolates from Burkina Faso without surface sterilization, two isolates of *B. bassiana s.l.* and three isolates of *S. lanosoniveum* significantly reduced the survival rates of *An. stephensi* compared with the control ($P < 0.001$). *B. bassiana s.l.* 99-1 showed the highest virulence of all isolates from Burkina Faso without surface sterilization. The MST ranged from 8.6 to 12.1 days for adults treated with fungi and was 17.0 days for the control (Table 1-1).

In the preliminary bioassay of 23 fungal isolates from the surface-sterilized mosquitoes from Japan, 10 isolates of *B. bassiana s.l.*, four isolates of *Lecanicillium* spp., and one isolate of *I. fumosorosea* resulted in significantly reduced survival rates of *An.*

stephensi compared with the control ($P < 0.001$). Of these isolates, only *B. bassiana s.l.* A-9-3-3 killed more than 90 % of *An. stephensi* within 14 days of exposure. *B. bassiana s.l.* A-9-3-3 showed the highest virulence of all isolates from surface-sterilized mosquitoes from Japan. The MST values ranged from 6.3 to 16.2 days for the adults treated with fungi, with the MST value for the control being 18.1 days (Table 1-1).

In the infectivity assay of 36 fungal isolates from Japan without surface sterilization, only 27 showed infectivity against female *An. stephensi*, namely, 11 *B. bassiana s.l.*, five *I. farinosa*, one *P. carneus*, and 10 *Lecanicillium* spp. isolates. Hyphal growth and conidiation of these isolates were observed on the dead mosquitoes. Furthermore, *B. bassiana s.l.* 31-37, 58-11, 59-45, 60-2, and 63-22 and *Lecanicillium* sp. 56-1 showed higher sporulation rates (>50 %). *B. bassiana s.l.* 60-2 showed both the lowest survival rates and highest infectivity (80 %) of all isolates tested against *An. stephensi* (Fig 1-1).

In the infectivity assay of 10 fungal isolates from Burkina Faso without surface sterilization, nine isolates showed infectivity against female *An. stephensi*; namely, three *B. bassiana s.l.*, two *S. lanosoniveum*, one *I. farinosa*, and three *L. araneicola* isolates. *B. bassiana s.l.* 99-1 showed the highest infectivity (50 %) of all Burkina Faso isolates against *An. stephensi* (Fig 1-1).

In the infectivity assay of 23 fungal isolates from surface-sterilized mosquitoes from Japan, all dead mosquitoes exhibited hyphal growth. Of these isolates, *B. bassiana s.l.* A-9-1-11, A-9-2-1, A-9-3-3, A-9-6-2, A-9-15-1, and A-9-14-2 showed higher sporulation rates (>50 %). *B. bassiana s.l.* A-9-3-3 showed the highest infectivity (66.7 %) of all isolates against *An. stephensi* (Fig 1-1).

I-3.6. Bioassay of selected isolates that showed higher virulence and/or infectivity against female adult *An. stephensi*

Compared to the control, all the isolates showed significantly higher mortality against *An. stephensi* ($P < 0.001$). The MST values ranged from 5.8 to 12.5 days for the isolates from Japan without surface sterilization (Table 1-2, Fig 1-2), 9.0 to 14.3 days for the isolates from Burkina Faso without surface sterilization (Table 2 & Fig 3), and 6.7 to 14.9 days for the isolates from surface sterilized mosquitoes from Japan (Table 1-2, Fig 1-4). In comparison, the MST value of the control was 17.0 days. In particular, *B. bassiana s.l.* 60-2 showed the highest virulence of all isolates against *An. stephensi* (Table 1-2).

I-4. Discussion

This study confirmed that it is possible to isolate various entomopathogenic fungi from adult mosquitoes collected under natural conditions (Table 1-3-5). Scholte et al. (2004) stated that various entomopathogenic fungi infect the larval stage of mosquitoes belonging to the Culicidae family in the field. However, only a few studies have reported the natural occurrence of major species of entomopathogenic fungi in or on adult mosquitoes that could be used as biocontrol agents. For instance, Pinnock et al. (1973) reported the natural occurrence of *B. bassiana* on adult *Cx. tarsalis*, *Cx. pipiens*, and *An. albimanus* collected from the field. Our results indicated that many of the fungal isolates obtained from wild mosquitoes exhibit both pathogenicity and infectivity against *An. stephensi*, particularly *B. bassiana s.l., Lecanicillium* spp., *I. farinosa*, and *I. fumosorosea* (Table 1-2, Fig 1-1). Furthermore, the latent infection rate of active mosquitoes (captured live with hand nets) collected in Japan was 4.7 %. Thus, the present study demonstrated that at least some species of entomopathogenic fungi adhered to or infected adult mosquitoes under natural conditions.

In addition, a number of minor species of entomopathogenic fungi were isolated in the present study, including *S. lamellicola*, *S. lanosoniveum*, *L. araneicola*, and *P. carneus* (Table 3). *S. lamellicola* is known to be a candidate entomopathogenic fungus for the biological control of ticks and scale insects (Zare and Gams, 2001), while *S.*

lanosoniveum has been isolated from *Proctolaelaps* sp. (Bałazy et al., 2008). *L. araneicola* has been isolated from species belonging to the order Araneae in Indonesia (Sukarno et al., 2009). Furthermore, *P. carneus* has potential ovicidal activity against *Ae. aegypti* (Luz et al., 2007). Our results provide new information showing that these fungal species also have the potential to infect adults of species from the Culicidae family.

The fungal isolates from Burkina Faso without surface sterilization tended to have lower abundance and species diversity than the isolates from Japan without surface sterilization (Table 1-3, 4). Wild mosquitoes that were collected at the same time and from the same region in Burkina Faso were mixed in the same tube. Consequently, 66 suspensions were made. Seventy-seven isolates of *L. araneicola* were obtained from a single suspension that contained 28 individual mosquitoes (a total of 1 685 mosquitoes collected in Burkina Faso were used in this experiment). It is likely that all 77 isolates originated from just one or a few individuals. Therefore, although it appears that *L. araneicola* occupancy might be common among all fungal isolates from Burkina Faso based on this overall result, *L. araneicola* may not, in fact, be a dominant species in this region, considering the small number of individuals that this species has actually infected. In addition, all isolates of *L. araneicola* showed relatively low virulence, although they did show some infectivity against *An. stephensi* based on the results shown in (Fig 1-1).

One reason for this difference might be the geographical location and extreme climatic conditions of Burkina Faso. For instance, the region has a mean maximum temperature of 35.9 °C and a mean total rainfall range of 0.1–1.2 mm month^{-1} during the dry season (World Weather Information Service, World Meteorological Organization <http://worldweather.wmo.int/143/c01532.htm>). Such dry weather conditions are considered unfavorable for generating an abundance and richness of fungi (Talley et al., 2002). Under such extreme environmental conditions, fungal survival and/or reproduction becomes difficult, thus reducing their chance of infecting insects. Another reason for this difference between the two locations is the sampling method that was implemented at Burkina Faso. The sampling method was conducted as part of the routine work of the Centre National de Recherche et de Formation sur le Paludisme, Burkina Faso. It is well known that some chemical insecticides drastically reduce conidial germination. For instance, some insecticides cannot be applied together with entomopathogenic fungi (e.g., Rashid et al., 2010). Hence, the spray catch method might have a negative impact on the isolation of fungi.

The fungal isolates from Japan and Burkina Faso without surface sterilization exhibited different levels of virulence (Fig 1-2, 3). Futuyma (1973) argued that species diversity, niche adaptation, and the specialization generated by the co-evolution of certain

species occur to a lesser extent in tropical than in temperate environments. In general, *Metarhizium* spp. exhibit high host specificity in tropical-subtropical regions (Bridge, 1997, Leal et al,, 1997 and St. Leger, 1992). While information about entomopathogenic fungi remains scarce for Burkina Faso, the environmental conditions of this region might have led to an evolutionarily stable intermediate level of virulence (Alizon et al., 2009).

Some entomopathogenic fungi were isolated from puddle water at one of the mosquito breeding sites in Japan. The conidia of *B. bassiana* are hydrophobic, thus, they float on the water surface and come in to contact with mosquito larvae that feed just below the surface, mainly at the tip of the siphon and head (Miranpuri and Khachatourians, 1991). In addition, the bodies of emerging adult *An. stephensi* mosquitoes come in contact with the water surface. These results show that it is highly likely that entomopathogenic fungi commonly occur under natural conditions and have the opportunity to adhere to and/or infect mosquitoes at breeding sites.

In this study, *B. bassiana s.l.* 60-2 showed the highest virulence (MST: 5.8 days). Fungal isolates showing MST values of 5–8 days against mosquitoes have been classified as high-virulence isolates in previous studies (Achonduh and Tondje, 2008, Howard et al., 2010 and Scholte et al., 2006). In the present study, several isolates of *Beauveria* spp. killed most of the *An. stephensi* individuals within 2 weeks, which is shorter than the

extrinsic incubation period (EIP) of the malaria parasite. This period represents the time from the infection of an arthropod insect vector to the time of infection of the next vertebrate host by the vector. It is important to incapacitate mosquitoes within this period to decrease the risk of transmitting malaria (Billingsley, 2010). Furthermore, *B. bassiana* has both lethal and pre-lethal effects on *An. stephensi* mosquitoes, such as reducing malaria transmission rates (Blanford et al., 2011). Furthermore, *Beauveria* spp. are accessible organisms, because they are easily isolated from various insect bodies or the soil by using simple media, antibiotics, or selective agents (Beilharz et al., 1982 and Chase et al., 1986). In addition, this group of species exhibits wide variation in their virulence towards different insect hosts. Therefore, *Beauveria* spp. have the potential to serve as practical entomopathogens for the biological control of vector mosquitoes. The results of the present study indicate that wild adult mosquitoes host various entomopathogenic fungi, which have applied potential for controlling mosquitoes acting as vectors of disease transmission.

The pathogenicity of fungal entomopathogens involves four steps: adhesion, germination, differentiation, and penetration. Although successful infection primarily depends on the adherence and penetration ability of a fungus to the host integuments, entomopathogenic fungi vary considerably in their mode of action and virulence (Shahid

et al., 2012). Furthermore, Ment et al. (2012) demonstrated that different fungus–host pairs are contributory factors to the level of host resistance and insecticidal mechanisms. Therefore, we intend to focus future studies on the insecticidal mechanisms and infection dynamics between *Beauveria* spp. and anopheline mosquitoes. These findings are expected to be important for the establishment of effective and convenient methods for vector control by using entomopathogenic fungi.

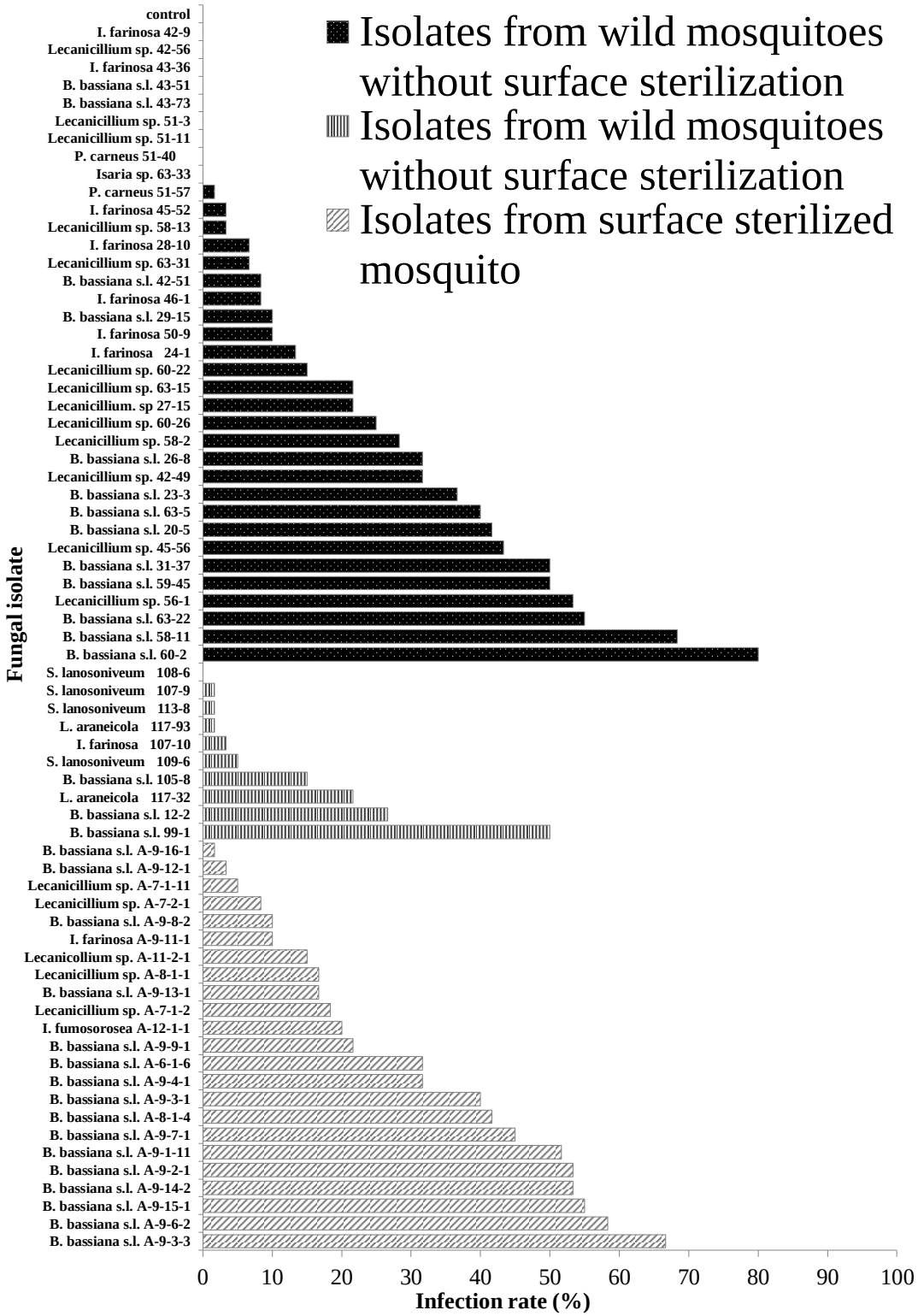

Fig. 1-1 - Sporulation rate of 69 fungal isolates in the preliminary examination.

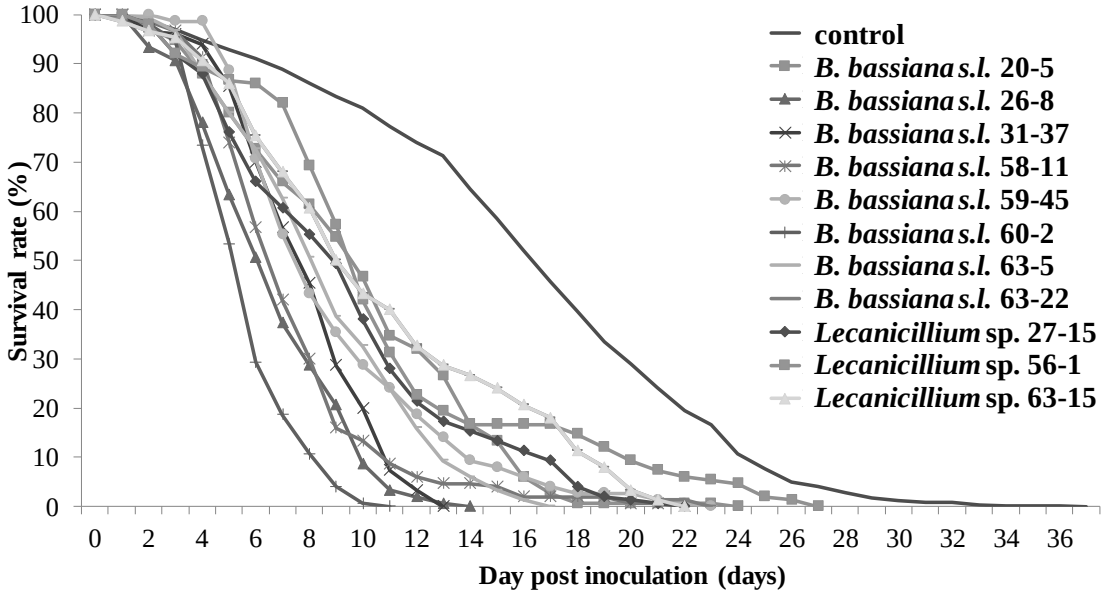

Fig. 1-2 - Survival rate of *An. stephensi* exposed to different isolates of entomopathogenic fungi from Japan without surface sterilization. Mosquitoes were exposed to isolates of *B. bassian*a sensu lato and *Lecanicillium* spp., and their survival was checked until all individuals were dead.

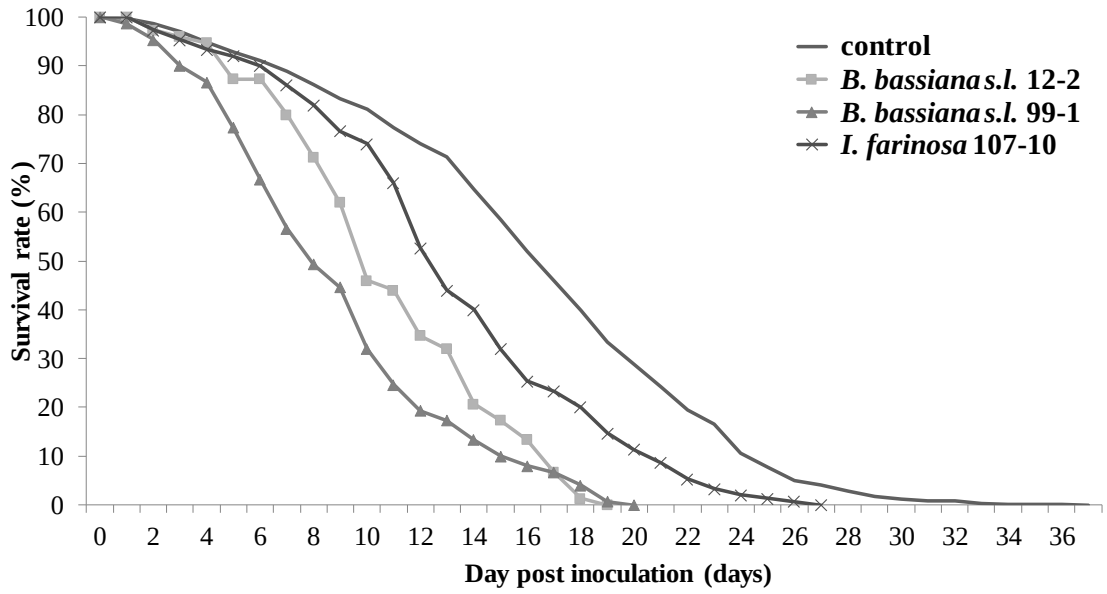

Fig. 1-3 - Survival rate of *An. stephensi* exposed to different isolates of entomopathogenic fungi from Burkina Faso without surface sterilization. Mosquitoes were exposed to isolates of *B. bassiana sensu lato* and *Isaria farinosa*, and their survival was checked until all individuals were dead.

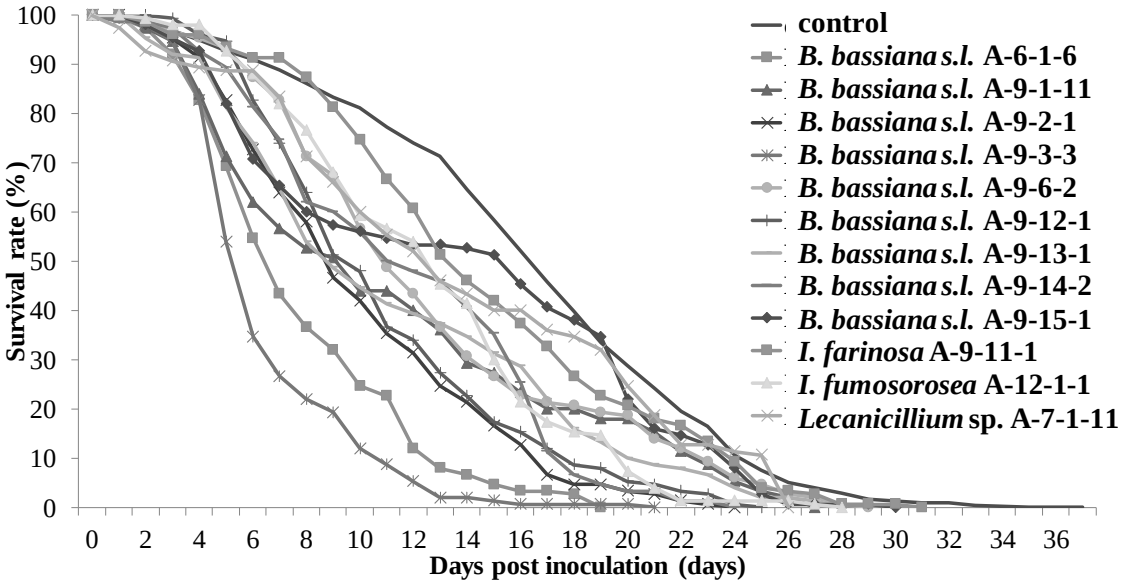

Fig. 1-4 - Survival rate of *An. stephensi* exposed to different isolates of entomopathogenic fungi obtained from surface-sterilized mosquitoes from Japan. Mosquitoes were exposed to isolates of *B. bassiana sensu lato*, *Isaria fumosorosea*, and *Lecanicillium* sp., and their survival was checked until all individuals were dead.

Table 1-1

Table 1- List of entomopathogenic hypocrealean fungi isolated from wild mosquitoes without surface sterilization collected in Hokkaido, Jap

Fungal species	Number of isolates	Host	Collection method	Date (month)
Beauveria bassiana sensu lat	2	*Aedes* spp.	CDC-LT*	June
B. bassiana s.l.	3	*Aedes* spp.	Netting	June
B. bassiana s.l.	15	*Aedes* spp.	Netting	July
B. bassiana s.l.	35	*Aedes* spp.	Netting	August
B. bassiana s.l.	30	*Aedes* spp.	Netting	September
Isaria farinosa	4	*Culex* spp.	CDC-LT	June
I. farinosa	19	*Aedes* spp.	Netting	July
I. farinosa	6	*Aedes* spp.	Netting	September
Isaria sp.	1	*Aedes* spp.	Netting	August
Lecanicillium araneicola	5	*Aedes* spp.	Netting	July
L. araneicola	3	*Aedes* spp.	CDC-LT	August
L. araneicola	2	*Aedes* spp.	Netting	August
L. araneicola	4	*Aedes* spp.	Netting	September
Lecanicillium sp.	1	*Aedes* spp.	CDC-LT	June
Lecanicillium spp.	16	*Aedes* spp.	Netting	July
Lecanicillium spp.	3	*Aedes* spp.	CDC-LT	August
Lecanicillium spp.	26	*Aedes* spp.	Netting	August
Lecanicillium spp.	49	*Aedes* spp.	Netting	September
Paecilomyces carneus	2	*Aedes* spp.	Netting	July
P. carneus	1	*Aedes* spp.	CDC-LT	August
P. carneus	1	*Aedes* spp.	Netting	September
Simplicillium lamellicola	1	*Aedes* spp.	Netting	July
S. lamellicola	5	*Aedes* spp.	Netting	August
S. lamellicola	2	*Aedes* spp.	Netting	September
S. lanosoniveum	1	*Aedes* spp.	Netting	August

*Centre for Disease Control Light Trap

Table 1-2

Table 2 - List of entomopathogenic hypocrealean fungi isolated from wild mosquitoes without surface sterilization in Goden, Burkina Faso

Fungal species	Number of isolates	Host	Collection method	Date (month)
Beauveria bassiana sensu lat	1	*Anopheles gambiae*	Spray catch	November. 2008
B. bassiana s.l.	2	*A. gambiae*	Spray catch	November. 2009
Isaria farinosa	1	*A. gambiae*	Spray catch	November. 2009
Lecanicillium araneicola	77	*A. gambiae*	Spray catch	November. 2009
Simplicillium lamellicola	13	*A. gambiae*	Spray catch	November. 2009

Table 1-3

Table 3 - List of entomopathogenic hypocrealean fungi isolated from surface sterilized mosquitoes collected in Hokkaido, Japan, 2009

Fungal species	Number of isolates	Host	Collection method	Date (month)
Beauveria bassiana sensu lat	8	*Aedes* spp.	Netting	August
B. bassiana s.l.	32	*Aedes* spp.	Netting	August
Isaria farinosa	1	*Aedes* spp.	Netting	August
I. fumosorosea	1	*Aedes* spp.	Netting	August
Lecanicillium spp.	20	*Aedes* spp.	Netting	August
Lecanicillium spp.	2	*Aedes* spp.	Netting	August

Table 1-4

Table 4 - MST and mortality of the 69 isolates in the preliminary examination.

Isotates	Median survival time		Log rank statistic (significance compared to controls)	Mortality within 14 days	Host	Collection method
	MST (days)	95% CI		mortality ± SE (%)		
Japan isolates without surface sterilization						
***Beauveria* spp.**						
B. bassiana s.l. 60-2	6.2	5.99 - 6.49	101.33 ***	100.0 ± 0.0	*Aedes* spp.	Netting
B. bassiana s.l. 58-11	7.3	7.03 - 7.66	90.70 ***	95.7 ± 4.7	*Aedes* spp.	Netting
B. bassiana s.l. 26-8	7.4	7.14 - 7.75	35.05 ***	97.4 ± 2.8	*Aedes* spp.	CDC-LT
B. bassiana s.l. 63-5	7.5	7.13 - 7.91	43.53 ***	89.4 ± 11.8	*Aedes* spp.	Netting
B. bassiana s.l. 20-5	7.8	7.36 - 8.26	11.59 ***	74.5 ± 4.7	*Aedes* spp.	Netting
B. bassiana s.l. 31-37	7.8	7.46 - 8.08	81.61 ***	100.0 ± 0.0	*Aedes* spp.	Netting
B. bassiana s.l. 59-45	8.2	7.81 - 8.63	49.90 ***	91.5 ± 9.4	*Aedes* spp.	Netting
B. bassiana s.l. 63-22	8.3	7.81 - 8.73	12.03 ***	74.5 ± 9.4	*Aedes* spp.	Netting
B. bassiana s.l. 23-3	8.5	8.13 - 8.79	11.50 ***	78.7 ± 11.8	*Aedes* spp.	Netting
B. bassiana s.l. 42-51	10.3	9.89 - 10.66	25.52 ***	74.5 ± 4.7	*Aedes* spp.	Netting
B. bassiana s.l. 29-15	11.0	10.60 - 11.30	3.56	63.9 ± 7.0	*Aedes* spp.	Netting
B. bassiana s.l. 43-73	11.4	11.04 - 11.80	16.05 ***	44.7 ± 9.4	*Aedes* spp.	Netting
B. bassiana s.l. 43-51	17.2	16.79 - 17.66	1.52	10.6 ± 2.3	*Aedes* spp.	Netting
***Lecanicillium* spp.**						
Lecanicillium sp. 27-15	7.3	6.96 - 7.68	94.98 ***	100.0 ± 0.0	*Aedes* spp.	CDC-LT
Lecanicillium sp. 63-15	7.6	7.24 - 7.92	53.27 ***	100.0 ± 0.0	*Aedes* spp.	Netting
Lecanicillium sp. 60-26	8.5	8.10 - 8.95	13.17 ***	85.1 ± 7.1	*Aedes* spp.	Netting
Lecanicillium sp. 45-56	9.1	8.79 - 9.38	1.32	76.6 ± 7.0	*Aedes* spp.	Netting
Lecanicillium sp. 42-49	9.2	8.72 - 9.74	29.55 ***	57.4 ± 9.4	*Aedes* spp.	Netting
Lecanicillium sp. 58-2	10.1	9.48 - 10.70	13.45 ***	53.2 ± 4.7	*Aedes* spp.	Netting
Lecanicillium sp. 58-13	10.2	9.83 - 10.62	45.67 ***	51.1 ± 16.5	*Aedes* spp.	Netting
Lecanicillium sp. 56-1	14.0	13.48 - 14.48	2.26	53.2 ± 4.7	*Aedes* spp.	Netting
Lecanicillium sp. 63-31	14.9	13.44 - 15.41	1.25	55.3 ± 2.4	*Aedes* spp.	Netting
Lecanicillium sp. 60-22	16.1	15.69 - 16.59	0.41	6.4 ± 14.1	*Aedes* spp.	Netting
Lecanicillium sp. 42-56	17.8	17.17 - 18.45	1.23	-4.3 ± 7.0	*Aedes* spp.	Netting
Lecanicillium sp. 51-3	18.0	17.49 - 18.41	0.53	6.4 ± 14.1	*Aedes* spp.	Netting
Lecanicillium sp. 51-11	18.3	17.89 - 18.76	5.82 *	-2.1 ± 4.7	*Aedes* spp.	Netting
***Isaria* spp.**						
I. farinosa 50-9	10.9	10.50 - 11.34	36.74 ***	65.9 ± 9.5	*Aedes* spp.	Netting
I. farinosa 24-1	11.3	10.72 - 11.88	3.87	10.6 ± 14.1	Culex spp.	CDC-LT
I. farinosa 46-1	11.5	11.05 - 12.34	32.74 ***	27.6 ± 14.2	*Aedes* spp.	Netting
I. farinosa 28-10	11.8	11.46 - 12.14	3.33	10.6 ± 7.1	*Aedes* spp.	Netting
I. farinosa 45-52	12.4	12.24 - 12.82	39.33 ***	57.4 ± 9.4	*Aedes* spp.	Netting
I. farinosa 42-9	12.5	12.14 - 12.92	24.69 ***	31.9 ± 4.7	*Aedes* spp.	Netting
I. farinosa 43-36	16.0	15.37 - 16.60	0.48	31.9 ± 18.9	*Aedes* spp.	Netting
Isaria sp. 63-33	18.2	17.80 - 18.68	0.26	-2.1 ± 4.7	*Aedes* spp.	Netting
***Paecilomyces* spp.**					*Aedes* spp.	Netting
P. carneus 51-57	11.4	11.06 - 11.84	15.74 ***	55.3 ± 16.5	*Aedes* spp.	Netting
P. carneus 51-40	16.3	15.76 - 17.02	1.05	-6.3 ± 7.1	*Aedes* spp.	Netting
control	19.5	19.12 - 19.98	-	0.0	-	-
Burkina Faso isolates without surface sterilization						
***Beauveria* spp.**						
B. bassiana s.l. 99-1	8.6	8.23 - 8.99	36.41 ***	83.0 ± 9.4	*Anopheles gambiae*	Spray catch
B. bassiana s.l. 105-8	10.6	10.13 - 11.08	8.48 *	44.7 ± 9.4	*A. gambiae*	Spray catch
B. bassiana s.l. 12-2	10.8	10.41 - 11.14	17.52 ***	66.0 ± 9.4	*A. gambiae*	Spray catch
***Simplicillium* spp.**						
S. lanosoniveum 113-8	9.4	8.91 - 9.81	40.71 ***	55.3 ± 2.4	*A. gambiae*	Spray catch
S. lanosoniveum 107-9	9.5	9.00 - 9.95	7.57 *	63.9 ± 7.0	*A. gambiae*	Spray catch
S. lanosoniveum 109-6	10.1	9.62 - 10.29	17.83 ***	53.2 ± 4.7	*A. gambiae*	Spray catch
S. lanosoniveum 108-6	10.4	10.03 - 10.86	38.04 ***	61.7 ± 9.5	*A. gambiae*	Spray catch
***Isaria* sp.**						
I. farinosa 107-10	10.5	10.13 - 10.85	1.47	51.0 ± 2.3	*A. gambiae*	Spray catch
***Lecanicillium* spp.**						
L. araneicola 117-32	11.1	10.61 - 11.57	0.63	42.6 ± 7.1	*A. gambiae*	Spray catch
L. araneicola 117-93	12.1	11.70 - 12.52	7.64 *	38.3 ± 2.4	*A. gambiae*	Spray catch
control	17.0	16.64 - 17.43	-	0.0	-	-
Japan isolates from surface-sterilized mosquitoes						
***Beauveria* spp.**						
B. bassiana s.l. A-9-3-3	6.3	5.95 - 6.68	61.33 ***	95.7 ± 4.7	*Aedes* spp.	Netting
B. bassiana s.l. A-6-1-6	8.1	7.71 - 8.49	52.59 ***	87.3 ± 4.7	*Aedes* spp.	Netting
B. bassiana s.l. A-9-8-2	11.3	10.88 - 11.77	26.56 ***	46.8 ± 11.8	*Aedes* spp.	Netting
B. bassiana s.l. A-8-1-4	11.4	10.90 - 11.87	35.07 ***	53.2 ± 4.7	*Aedes* spp.	Netting
B. bassiana s.l. A-9-1-11	11.4	10.97 - 11.81	48.34 ***	44.7 ± 9.4	*Aedes* spp.	Netting
B. bassiana s.l. A-9-14-2	11.5	11.07 - 11.99	3.46	40.4 ± 9.4	*Aedes* spp.	Netting
B. bassiana s.l. A-9-15-1	11.9	11.31 - 12.50	4.59	40.4 ± 4.7	*Aedes* spp.	Netting
B. bassiana s.l. A-9-4-1	12.0	11.49 - 12.46	15.07 ***	57.4 ± 9.4	*Aedes* spp.	Netting
B. bassiana s.l. A-9-2-1	12.6	12.08 - 13.10	12.29 ***	46.8 ± 7.1	*Aedes* spp.	Netting
B. bassiana s.l. A-9-6-2	13.0	12.48 - 13.49	9.14 ***	53.2 ± 4.7	*Aedes* spp.	Netting
B. bassiana s.l. A-9-7-1	13.0	12.10 - 13.28	0.54	46.8 ± 16.5	*Aedes* spp.	Netting
B. bassiana s.l. A-9-16-1	13.3	12.80 - 13.73	12.84 ***	29.8 ± 16.5	*Aedes* spp.	Netting
B. bassiana s.l. A-9-9-1	13.4	12.85 - 13.94	6.22	44.7 ± 4.7	*Aedes* spp.	Netting
B. bassiana s.l. A-9-12-1	14.3	13.83 - 14.68	9.45 ***	29.8 ± 11.8	*Aedes* spp.	Netting
B. bassiana s.l. A-9-13-1	14.8	14.21 - 15.32	0.99	19.1 ± 9.4	*Aedes* spp.	Netting
B. bassiana s.l. A-9-3-1	15.1	14.54 - 15.65	0.84	25.5 ± 11.8	*Aedes* spp.	Netting
***Lecanicillium* spp.**						
Lecanicillium sp. A-7-1-2	11.6	11.16 - 12.09	44.45 ***	55.3 ± 2.4	*Aedes* spp.	Netting
Lecanicillium sp. A-7-1-11	12.2	11.84 - 12.65	48.10 ***	34.0 ± 7.1	*Aedes* spp.	Netting
Lecanicillium sp. A-8-1-1	12.4	11.75 - 12.96	23.76 ***	51.1 ± 7.1	*Aedes* spp.	Netting
Lecanicollium sp. A-11-2-1	12.5	12.09 - 12.88	19.75 ***	40.4 ± 9.4	*Aedes* spp.	Netting
Lecanicillium sp. A-7-2-1	16.0	15.50 - 16.55	8.40 *	14.9 ± 4.7	*Aedes* spp.	Netting
***Isaria* spp.**						
I. fumosorosea A-12-1-1	14.0	13.56 - 14.45	8.82 ***	25.5 ± 11.8	*Aedes* spp.	Netting
I. farinosa A-9-11-1	16.2	15.74 - 16.72	0.32	2.1 ± 9.4	*Aedes* spp.	Netting
control	18.1	17.91 - 18.48	-	0.0	-	-

$^{***}P < 0.001$, $^{**}P < 0.001$ and $^{*}P < 0.05$ (Log rank statistics, significance compared to controls))

Table 1-5

Table 5 - MST and mortality of selected isolates

Isotates	Median survival time		Log rank statistic (significance compared to controls)	Mortality within 14 days		Origin
	MST (days)	95% CI		mortality ± SE (%)		
Beauveria bassiana sensu lato 60-2	5.8	5.67 - 5.99	194.7	100.0 ± 0.00	a	Japan
B. bassiana s.l. A-9-3-3	6.7	6.44 - 6.88	180.6	96.8 ± 2.20	ab	Japan (surface sterilization)
B. bassiana s.l. 26-8	6.8	6.58 - 6.97	190.3	100.0 ± 0.00	a	Japan
B. bassiana s.l. 58-11	7.6	7.33 - 7.78	165.7	92.9 ± 4.92	ab	Japan
B. bassiana s.l. A-6-1-6	8.0	7.71 - 8.20	159.9	90.0 ± 4.41	abcd	Japan (surface sterilization)
B. bassiana s.l. 31-37	8.0	7.84 - 8.22	174.7	100.0 ± 0.00	a	Japan
B. bassiana s.l. 63-5	8.8	8.59 - 9.03	160.2	91.6 ± 4.23	abc	Japan
B. bassiana s.l. 99-1	9.0	8.75 - 9.27	136.0	79.4 ± 4.62	abcde	Burkina Faso
B. bassiana s.l. 59-45	9.1	8.89 - 9.37	137.0	86.3 ± 4.69	abcd	Japan
Lecanicillium . sp 27-15	9.7	9.41 - 9.97	108.3	73.2 ± 5.93	bcdef	Japan (surface sterilization)
B. bassiana s.l. 20-5	10.0	9.71 - 10.22	124.2	74.8 ± 6.28	bcdef	Japan
B. bassiana s.l. A-9-2-1	10.2	9.90 - 10.44	109.7	66.5 ± 7.09	defg	Japan (surface sterilization)
B. bassiana s.l. 63-22	10.8	10.52 - 11.09	90.8	58.6 ± 5.70	efgh	Japan
B. bassiana s.l. 12-2	10.9	10.67 - 11.17	109.2	68.2 ± 8.21	cdefg	Burkina Faso
Lecanicillium sp. 56-1	11.1	10.75 - 11.35	67.2	74.3 ± 4.54	bcdef	Japan
B. bassiana s.l. A-9-12-1	11.1	10.84 - 11.38	83.5	65.1 ± 5.19	hijk	Japan (surface sterilization)
B. bassiana s.l. A-9-1-11	11.2	10.79 - 11.51	55.1	54.0 ± 9.85	fghi	Japan (surface sterilization)
B. bassiana s.l. A-9-13-1	11.5	11.20 - 11.85	50.8	47.6 ± 6.03	ghji	Japan (surface sterilization)
Lecanicillium sp. 63-15	11.8	11.54 - 12.14	60.0	47.9 ± 6.90	ghji	Japan (surface sterilization)
B. bassiana s.l. A-9-14-2	11.9	11.60 - 12.16	75.2	35.2 ± 12.42	hijk	Japan (surface sterilization)
Isaria fumosorosea A-12-1-1	12.8	12.51 - 13.05	61.1	37.3 ± 3.65	hijk	Japan (surface sterilization)
B. bassiana s.l. A-9-6-2	13.1	12.78 - 13.43	29.0	50.8 ± 6.94	fghij	Japan (surface sterilization)
B. bassiana s.l. A-9-15-1	13.8	13.45 - 14.15	17.3	17.9 ± 11.97	kl	Japan (surface sterilization)
Lecanicillium sp. A-7-1-11	13.8	13.48 - 14.16	23.0	40.0 ± 7.41	hijk	Japan (surface sterilization)
I. farinosa 107-10	14.3	14.03 - 14.61	34.8	32.4 ± 9.43	ijk	Burkina Faso
I. farinosa A-9-11-1	14.9	14.56 - 15.16	17.8	27.7 ± 10.90	jk	Japan (surface sterilization)
control	17.0	16.73 - 17.31	-	0.0	l	-

All treatments are significantly different compared to controls according to Log rank static ($P < 0.001$).
In the same column, means followed by the same letters are not significantly different ($P < 0.01$).

I-5. Summary

We investigated the potential of entomopathogenic fungi that naturally occur in or on adult mosquitoes for use as biocontrol agents of vector mosquitoes. The fungi were isolated from wild mosquitoes collected in Japan and Burkina Faso using two isolation methods (with and without surface sterilization). Detected fungal species included *B. bassiana sensu lato*, *Isaria* spp., *Paecilomyces* spp., *Lecanicillium* spp., and *Simplicillium* spp. These isolates were used in bioassays against adult female *Anopheles stephensi* mosquitoes. The median survival time ranged from 5.8 to 14.9 days (control, 17.0 days). Reduced survival times were observed in the isolates from surface-sterilized mosquitoes from Japan, with the isolate *B. bassiana s.l.* 60-2 exhibiting the highest virulence. This study indicates that adult mosquitoes are naturally infected with various entomopathogenic fungi, and that some of these isolates have the potential for use as fungal pesticides to control vector mosquitoes.

Chapter II

An entomopathogenic fungus strain of *Beauveria bassiana* kills *Anopheles* mosquito by brain infection

II-1. Introduction

Entomopathogenic fungi, such as *B. bassiana* and *M. anisopliae*, are known to control mosquito populations, and have been studied extensively in field and laboratory (Blanford et al., 2010 and Scholte et al., 2005). Unlike other types of entomopathogens, such as bacteria, microsporidia, and viruses, fungal pathogens can infect and kill mosquitoes by percutaneous infection. Therefore, like chemical insecticides, they can be sprayed on the indoor surfaces of houses, cotton ceiling hangings, curtains, and bed nets (Scholte et al., 2005, Thomas and Read, 2007 and Achonduh et al., 2008). In these studies, vector mosquitoes, after feeding on blood, were exposed to pathogens through tarsal contact on the treated surface, during a resting period. Fungal infection by tarsal contact was shown to be sufficient in causing > 90% mortality (Blanford et al., 2005). In (Chapter II), I established an original fungal library originating from wild mosquitoes. Four hundred thirteen isolates of entomopathogenic fungi were isolated from wild mosquitoes collected in Japan and Burkina Faso; of these *B. bassiana s.l.* 60-2 showed the highest virulence, with a median survival time (MST) of 5.8 days, against *An. stephensi* using the tarsus topical inoculation method (Scholte et al., 2005, Thomas and Read, 2007 and Achonduh et al., 2008) and could, thus, be potentially used as a fungal bio-pesticide for controlling vector mosquitoes.

Entomopathogenic fungi generally exist in nature in soil, water, and plants and infect their insect hosts. Fungal infection begins when conidia attach to the cuticle of insects; subsequently, the spores germinate and penetrate into the cuticle. On reaching haemocoel, the fungus multiplies as hyphal bodies. After death of the host, the hyphae extrude from the interior to the exterior of the insect through the cuticle and produce conidia on the insect cadaver (Roberts, 1981). *B. bassiana* were observed to grow and reproduce primarily in the haemocoel of *Carposina sasakii*, and subsequently invade the internal tissues (e.g., fat body, muscle, Malpighian tubules, gut, and even the silk gland) (Xiong et al., 2013). The mycelium of *M. anisopliae* was observed to first colonize the fat body of *Diatraea saccharalis*, and subsequently the muscle tissue after the death of host[10]. In others studies, various infection dynamics between the fungus–host pairs have been reported (Liu et al., 2011, Schreiter et al., 1994 and Ment et al., 2012). Because their modes of interaction are very complex and specific, we need to investigate the infection dynamics between each fungus–host pair for establishing an efficient control method.

In previous studies, generally, the insect pests were inoculated with entomopathogenic fungi by spray or dip inoculation method (Inglis et al., 2012), by which the fungal conidia adhered to their entire body. The infection dynamics of entomopathogenic fungi have been investigated for such inoculation methods (Xiong et

al., 2013, Schneider et al., 2013, Liu et al., 2011, Schreiter et al., and Ment et al., 2012). The tarsus inoculation method for entomopathogenic fungi is an important method for controlling adult mosquitoes (Blanford et al., 2010 and Scholte et al., 2005). However, in contrast to the general inoculation methods (e.g. spraying or dipping) for entomopathogenic fungi, the mechanisms for infection and colonization of pathogens using the tarsus inoculation method have been less investigated. For instance, whether the infection starts from tarsus and then the fungus penetrates into the haemocoel of tarsus, or whether the fungal conidia initially adhere to the tarsus and then they are transmitted to the other body parts by grooming behaviour, is unknown. In the study involving *B. bassiana* and *An. gambiae s.l.*, using the tarsus topical inoculation method, it was observed that the fungal conidia attached to and germinated on the mouth part, thorax, and forelegs before eventually spreading to other parts of the insect (Achonduh et al., 2008); moreover the spread of the fungus was only on the mosquito body surface. The clarification of the infection mechanism using the tarsus topical inoculation method would facilitate more effective control of vector mosquitoes. Therefore, it is important to understand the infection dynamics of *B. bassiana s.l.* 60-2 in adult mosquitoes.

In the present study, the infection dynamics of *B. bassiana s.l.* 60-2 in *An. stephensi* was investigated by histopathological observation using Grocott stain method.

The understanding of infection dynamics between *B. bassiana s.l.* 60-2 and *An. stephensi* might be one of the steps in elucidating infection mechanism, and might facilitate the mosquito control.

II-2. Materials & Methods

II-2.1. Fungus

B. bassiana s.l. 60-2 was isolated from wild mosquitoes in Japan, and showed highest virulence against *An. stephensi*, in (Chapter I). The fungal isolate was cultured on a 90-mm potato dextrose agar plate at 24°C in the dark for 10–15 days. The germination rate of the fungal isolate was more than 99.2%.

II-2.2. Mosquitoes

The larvae of *An. stephensi* were reared for approximately 10 days in plastic trays (20.5 × 26.5 × 4.5 cm). The larvae were fed Hikari Economy® (KYORIN CO., LTD., Japan) fish food daily. The pupae were collected daily and transferred to mesh cages (27 × 27 × 27 cm). The adults that emerged were fed 10% (w/v) sucrose/water solution *ad libitum*. During all the developmental stages, mosquitoes were kept in an incubator at 27°C with

16:8 h light:dark photoperiod. For the bioassay, 4–6-days-old adult female non blood-feeding mosquitoes were transferred from the adult cage to an acrylic bottle (2.7 cm diameter × 12 cm height) using a mouth aspirator.

II-2.3. The tarsus topical inoculation

The spore concentration was adjusted to 1.3×10^7 conidia ml^{-1} in 0.05% Tween 20 solution with the help of a haemocytometer. One millilitre of this suspension was evenly pipetted over a 90-mm-diameter filter paper, resulting in conidial densities of 2.0×10^{10} conidia/m^2. For control, 1 ml of 0.05% Tween 20 solution was pipetted evenly over a filter paper. This filter paper was then placed in the lid of a 90-mm-diameter Petri dish.

Thirty mosquitoes were transferred from the adult cage to an acrylic bottle and were anesthetized by carbon dioxide gas. The mosquitoes were transferred on the filter paper in an exposure Petri dish, prepared as described in the previous paragraph, and were placed in direct contact with the fungal inoculum. After recovery from the anaesthesia, the mosquitoes were allowed to move on the inoculum for 30 min. After the exposure, the mosquitoes were transferred to bottom-meshed assay tubes (8.5 cm in diameter and 9.5 cm in height), which were covered with nylon socks with the toe part cut off. This inoculation method used is described elsewhere (Farenhorst and Knols, 2010). The

mosquitoes were kept at 27 ± 1°C, 80 ± 4% relative humidity, and 16:8 h light:dark photoperiod. They were fed 10% (w/v) sucrose/water solution *ad libitum*. The mortality was monitored daily, until all the mosquitoes were dead.

II-2.4. Identification of the fungal adhesion part on mosquito

Thirty mosquitoes were dissected immediately after the inoculation using forceps under light microscope (Leica: DMI 3000B) at 10X magnification. These body parts (head, thorax, wings, legs, and abdomen) were placed in the modified entomopathogenic fungi selective medium consisting of 10 g glucose, 10 g peptone, 15 g oxygal, 60 mg rose Bengal, 0.5 g chloramphenicol, 10 mg dodine, 0.25 g cycloheximide, 60 mg streptomycin, 60 mg penicillin G potassium, and 20 g agar per litre sterile distilled water[32]. The fungal growth on the medium was observed to evaluate the adhesion rates of each part 3 days after inoculation. This experiment was conducted in triplicates.

II-2.5. Histopathological observation of infection dynamics

A total of 210 mosquitoes (seven treatments; 30 mosquitoes per treatment) were exposed to *B. bassiana s.l.* 60-2 by tarsus topical inoculation, and then 30 mosquitoes each were transferred to seven bottom-meshed assay tubes. On 1, 2, 3, 4, 5, 6, and 7 days after

inoculation, 30 mosquitoes were collected in one of the bottom-meshed assay tube, and control mosquitoes were collected only 7 days after the treatment. The dead mosquitoes were placed in Bouin's fixative (75 ml containing picric acid saturated aqueous solution, 25 ml formaldehyde, and 1 ml glacial acetic acid) at 60°C for 10 min, and then kept at room temperature for 24 h. These were then washed thrice with 70% ethanol and dehydrated using a graded series (70%, 95%, and three times in 100%) of ethanol for 1 h each and paraffinized sequentially in xylene I and II for 30 min, each. Before the paraffinization, the bodies of mosquitoes were kept in the mixture of approximately 50% xylene and 50% paraffin for 30 min and then dipped thrice in pure paraffin solution overnight in an incubator at 60° C. The mosquito bodies embedded in paraffin were sectioned into 5–20 µm slices (Leica: SM2010R) and dried on slides at 37°C. These slides were incubated overnight at 56°C before deparaffinization in xylene and were rehydration in a graded ethanol series. The method used for making the paraffin section was a modification of the method reported in literature (Wang et al., 2010, Farnesi etal., 2012 and Nagata et al., 1999). Finally, the sections of the infected and uninfected mosquito bodies were stained, using fast green for mosquito bodies and Grocott stain for fungi (Grocott, 1955), and observed under light microscope (Leica: DMI 3000B).

The paraffin sections of 10 randomly selected mosquitoes from the 30

mosquitoes of each group, 1, 2, 3, 4, 5, 6, and 7 days after inoculation as well as of the control group were evaluated for determining the fungal invasion rate in each body part and tissue. The head, thorax, and abdomen of each specimen were observed and the fungal invasion rates in each part were analysed by confirming the presence of fungal propagules. Furthermore, the specimen that showed invasion to the head were analysed for calculating the invasion rate to the brain. This experiment was conducted in triplicates.

II-2.6. Comparing fungal invasion rate of alive and dead mosquitoes

A total of 300 mosquitoes (10 treatments; 30 mosquitoes per treatment) were exposed to *B. bassiana s.l.* 60-2 by tarsus topical inoculation, and 30 mosquitoes were transferred to the bottom-meshed assay tubes. After fungal inoculation, the alive and dead individuals (more than 10 mosquitoes, each) were collected daily. The alive individuals were collected daily up to 7 days after the inoculation. The dead individuals were collected daily from 3 to 7 days after the inoculation (because mosquitoes did not die 1 and 2 days after the inoculation in this experiment). The paraffin sections of 10 randomly selected individuals from among the alive and dead individuals on each day after inoculation were evaluated to detect the fungal invasion rate to each part as described in the previous section. The fungal invasion rates of the alive and dead individuals were compared. This

experiment was conducted with triplicates.

II-2.7. Comparison of the survival rates in the different infection routes through proboscis and tarsus

The spore concentration was adjusted to 1.3×10^7 conidia/ml in 0.05 % Tween 20. Thirty mosquitoes were transferred to an acrylic bottle (8.5-cm diameter and 9.5-cm height) from the adult cage, and anesthetized by carbon dioxide gas. The proboscis or tarsus of mosquitoes were inoculated using a paintbrush. The inoculated mosquitoes were kept in the same condition as described in the section above. After inoculation, the survival rates were monitored daily, until all the mosquitoes were dead. This experiment was conducted in triplicates.

II-2.8. Statistical analysis

The adhesion rates of conidia and the fungal invasion rates for each part on each day after the inoculation were analysed using ANOVA. When the F test was significant at $p < 0.01$, the treatment means were compared using Tukey's Honest Significant Difference test. The correlation coefficient between the fungal invasion rate and mortality was analysed using Pearson's product–moment correlation. For all the treatments, the median survival

times were determined using Kaplan–Meier survival analyses, with significant differences between the treatments and controls estimated using a log rank test. The Abbott's formula was used to correct for the natural mortality in all the bioassays. The arcsine transformation was used for all the percentage datasets.

II-3. Results

II-3.1. Fungal infection occurs on the tarsus and proboscis by the tarsus inoculation method

The fungal development on legs, proboscis, abdomen, and wings was observed. Although high rates of fungal development were observed on legs and proboscis (99 and 97%, respectively), fungal growth was also observed on abdomen and wings at the rates of 13 and 17% (n = 90), respectively (Fig. 2-1). The fungal adhesion rates on legs and proboscis were significantly higher than those on the other parts ($p < 0.01$).

II-3.2. *B. bassiana s.l.* 60-2 invades various organs and tissues in early stage of infection

Grocott stain is useful for distinguishing the fugal hyphae because they are specifically stained black in contrast to the host tissues and organs which are stained in different

colours (e.g., fast green). In control, the head, thorax, and abdomen parts were clearly observed in *An. stephensi* without fungal inoculation (Fig. 2-2 A-C). On the other hand, fungal propagules were observed in infected *An. stephensi* from 3 days after inoculation; these propagules multiplied in the haemocoel of the host, and fungal invasion to the tissues and organs was observed from 3 days after inoculation (Fig. 2-2 D-F). The fungal propagules were observed in almost all the tissues and organs 7 days after inoculation in almost all the individuals (Fig. 2-2 G-I). The extension and invasion of the fungal propagules were observed not only in the haemocoel but also in various tissues and organs, such as compound eyes, brain, salivary glands, maxillary palpus, dorsal longitudinal muscle, midgut, ovary, and Malpighian tubules in the early stage of infection (Fig. 2-3).

II-3. 3. Fungal invasion to the brain correlated with mosquito mortality

The initial fungal invasion was observed in proboscis (especially, in labrum and labium) and tarsus (Fig. 2-4 B, F), and then the fungal propagules invaded the base of proboscis in the head and the base of legs in the thorax (Fig. 2-4 D, H) at 3 days after inoculation. The fungal invasion rate to the head was significantly higher compared to that in the other parts at 3 days after inoculation ($n = 30$, $p < 0.05$). The fungal invasion rate to the head did not show significance at 4 days after inoculation, but that in the head tended to be

higher than in the other parts on all the days (Fig. 2-5). Furthermore, the fungal invasion rate in the head parts showed higher correlation with mortality ($r = 0.911$, $t = 9.668$, $df = 19$, $p < 0.01$) compared to the invasion rate in the thorax and abdomen parts (Table 2-1). Accordingly, we focused on the correlation between fungal invasion to the brain and mortality because the rate of fungal invasion to the head was higher than to the other parts and it was known that many species of parasite infect the host brain. Correlation coefficient between the fungal invasion rate to the brain and mortality showed a higher value ($r = 0.989$, $t = 29.041$, $df = 19$, $p < 0.01$) compared with the other parts (Table 2-1).

II-3. 4. Fungal invasion to the brain causes mosquito death

The infected alive and dead individuals were examined separately, and the fungal invasion rates of each part were evaluated at 1 to 7 days after inoculation. In the live individuals, the fungal invasion rate to the head was significantly higher than to the other parts at 3 days after inoculation ($n = 30$, $p < 0.05$), and the fungal invasion to the head and thorax was significantly higher than the invasion to abdomen at 4 days after inoculation ($n = 30$, $p < 0.05$). Although fungal invasion to the brain was detected in all the dead individuals (Fig. 2-6 B, D and F), it was not detected in any alive *An. stephensi* (Fig. 2-6 A, C and E). The fungal propagules multiplied each day, and the fungal hyphae invaded to almost of

all the tissues and organs of *An. stephensi* before or after host death (Fig. 2-6). On the other hand, fungal invasion to the brain was observed in only the dead individuals on any day post inoculation (Fig. 2-7).

II-3. 5. Early death of mosquitoes occurred only through the proboscis route of infection

The MST values were 6.5 days for the infection route from proboscis and 11.3 days for the infection route from tarsus. In comparison, the MST value of the control was 18.1 days. The early death of adult mosquitoes occurred only when the route of infection was from the proboscis (Fig. 2-8). The infection route from the proboscis and tarsus showed significantly lower survival rates compared that for control, according to the log rank test ($n = 90$, $p < 0.001$). The infection route from the proboscis showed significantly lower survival rate compared to the infection route from the tarsus according to log rank test ($n = 90$, $p < 0.001$).

II-4. Discussions

To use *B. bassiana s.l.* 60-2 more effectively for the control of vector mosquito, we need

to understand the property and infection dynamics of this entomopathogenic fungi in *Anopheles* mosquito. Generally, once the entomopathogenic fungi penetrate into the host integument, the hyphal bodies multiply in the haemocoel in the early stage of infection, and then fungal hyphae invade some tissues or organs at the latter stage of infection. After the death of the host, fungal hyphae invade all the tissue and organs (Liu et al., 2011, Schreiter et al., 1994, Chouvenc et al., 2009 and Toledo et al., 2010). In contrast, the present study showed that fungal adhesion occurred on the leg parts and the proboscis in the tarsus inoculation method (Fig. 2-1), and the fungal hyphae were first developed on the leg parts and the proboscis (Fig. 2-4). The fungal hyphae were observed not only in the haemocoel but also in various other tissues or organs in the early stage of infection (Fig. 2-2, 3). Furthermore, it was observed that fungal hyphae of *B. bassiana s.l.* 60-2 invaded almost all the tissues and organs of *An. stephensi* before and after the host death.

Some pathogens have a predilection for infecting the head part of the host, including the brain and/or central nervous system (CNS). Baculovirus infect the host brain by using a protein tyrosine phosphatase and then induce enhanced locomotory activity of the Lepidopteran host (Katsuma et al., 2012). In the fungal pathogen, *Ophiocordyceps unilateralis s.l.*, a high-density of hyphal body was observed in the head of the host ant, *Camponotus leonardi*, before their death (Hughes et al., 2011). This

phenomenon was thought to be the strategy of the parasite to manipulate the behaviour of the host before dying. *O. unilateralis s.l.* was reported to specifically invade the brain tissues of its naturally occurring host ant, *C. leonardi* (Bekker et al., 2014). The present study showed that fungal invasion rate to the head part was higher than to any other part (Fig. 5). It is known that *B. bassiana* and *M. anisopliae* affect some behaviour of their hosts; They cause reduced searching and frequency of blood feeding in *An. gambiae* and *An. Stephensi* (George et al., 2011, Mnyone et al., 2011, Scholte et al., 2006, Blanford et al., 2011). There is some possibility that such behavioural changes might be induced by fungal infection in the head part of the host including brain and/or CNS.

The lethal factor of entomopathogenic fungi has been discussed in various studies. Schneider et al. (2013) concluded that the death of host occurred by fungal invasion to some organs of the host and probably by a fungal toxin. Death of an insect is often due to a combination of histolytic action, mechanical blockage of the gut by the mycelium, damaging effect of the physical presence of the mycelium, and toxin production in the hemolymph (Mc Cauley et al., 1968). It has been discussed that entomopathogenic fungi have various factors for killing a host, such as a mycotoxin, physiological disturbance and starvation (Kershaw et al., 1999). In addition, it was demonstrated that different fungus–host pairs are the contributing factors to the level of

host resistance and for the insecticidal mechanisms, as described by Ment *et al*. (2012). In the present study, the fungal invasion rate to the head was higher than in the other parts (Fig. 2-6), and the fungal invasion to the brain was clearly correlated with the mortality (Table 1). This is the first study on mosquito control, which reveals that fungal invasion to the brain affects the host death. Although there are various studies on lethal factors for host insects upon entomopathogenic fungi infection, it has not been reported that invasion to the brain caused the host death. This phenomenon has often been observed for other pathogen–host pairs. The invasion of West Nile Virus into the brain plays a crucial role in neurological damage and induces dysfunction of CNS cells or directly causes death (Xiao et al, 2001 and Xianli et al., 2010). Fungal pathogens, such as *Ochroconis gallopavum*, *Scopulariopsis brumptii*, and *Cladophialophora bantiana* were reported to cause brain abscesses and killed the human host (Singh et al., 1997). In the present study, all the dead individuals were infected in their brain by *B. bassiana s.l.* 60-2. In contrast, fungal invasion was not detected in the brain until 7 days after inoculation in all the alive individuals (Fig. 2-6, 7). It was suggested that *B. bassiana s.l.* 60-2 invasion to the brain directly caused the death of its host, *An. stephensi*.

The MST value for infection through the proboscis route was lower than for the tarsus route (Fig. 2-8). It appears that complex factors, such as the physical distance from

the brain and immune reaction of the host, are responsible for this result. Initially, the fungal infection occurred on the leg parts and proboscis (Fig. 2-1), and then the fungal hyphae invaded the thorax and head parts, respectively (Fig. 2-4). Fungal invasion to the brain is one of the causes for the mosquito death. Through the proboscis route, the fungus could invade the brain earlier compared to infection through the tarsus route because of the physical distance involved. In contrast, some studies have been discussed the effects of host immunity in this regard. In a study on the immune response of *An. gambiae*, more haemocytes were observed to exist in the thorax part than in the head part, leg, maxillary palps, and mouth parts (King and Hillyer., 2013). In the infection through the tarsus route, the fungal hyphae invaded the head part and the brain via the thorax part. Therefore, the fungal invasion to the brain through the tarsus route of infection might be inhibited by more number of haemocytes in the thorax parts, and the tarsus route infection caused slow death compared to the proboscis route infection (Fig. 2-8). The lethal factor is not limited to just fungal invasion to the brain. In fact, in some of the cases in the present study, fungal invasion to the brain was not detected in the dead individuals in tarsus route infection (date not shown). As described above, there are a lot of other lethal factors (e.g., mycotoxin and enzyme activity), and insect death must be a result of coaction of these factors (Kershaw et al., 1999 and Lu et al., 2015). The dead individuals without brain

infection in the tarsus infection route could be killed by the effects of these factors. Moreover, the proboscis route infection also caused mosquito death, but especially, the fungal invasion to the brain from the proboscis route infection triggered earlier death compared to the tarsus route infection. It was revealed that the fungal invasion to the brain induced mosquito death, and thus, the proboscis route infection is important for controlling the vector mosquitoes.

In conclusion, by tarsus topical inoculation method, *B. bassiana s.l.* 60-2 conidia adhered to the leg parts and proboscis; the fungal invasion to the brain caused "early death" of the mosquitoes only in the proboscis route infection. These results show that the fungal invasion to the brain and proboscis route infection are important to control the vector mosquitoes. In our previous study, virulence of *Beauveria* spp. against *An. stephensi* was observed to be significantly different between the fungal isolates (with MST values ranging from 5.8 to 17.2 days) in (Chapter I). Although, this mode of action for the killing of mosquitoes might be specific for *B. bassiana s.l.* 60-2, other isolates of *B. bassiana s.l.*, including those with lower virulence, should be investigated for the same. Our results should contribute in establishing a new vector biocontrol approach by providing a more effective inoculation method for mosquito proboscis.

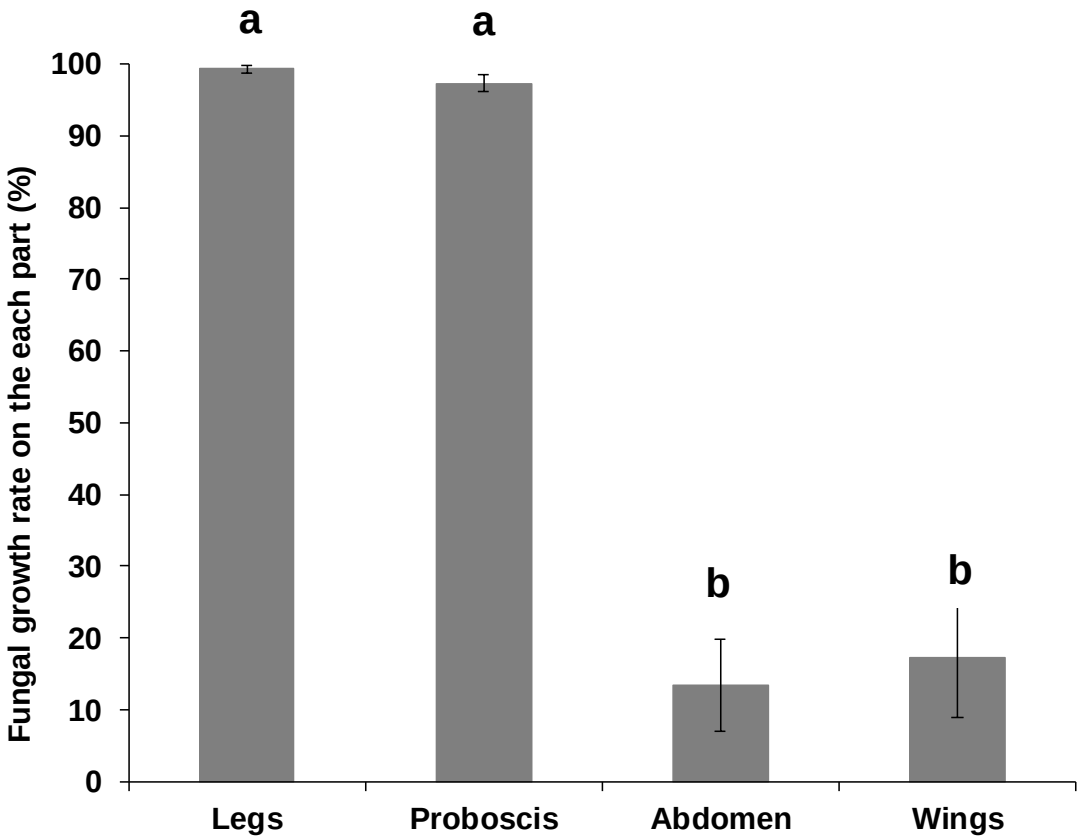

Figure 1. Mean percentage of fungal growth on each mosquito body parts. Error bars represent the standard error of the mean (n=90). Different letters indicate significant differences among body parts ($p < 0.01$, Tukey's honest significant difference test).

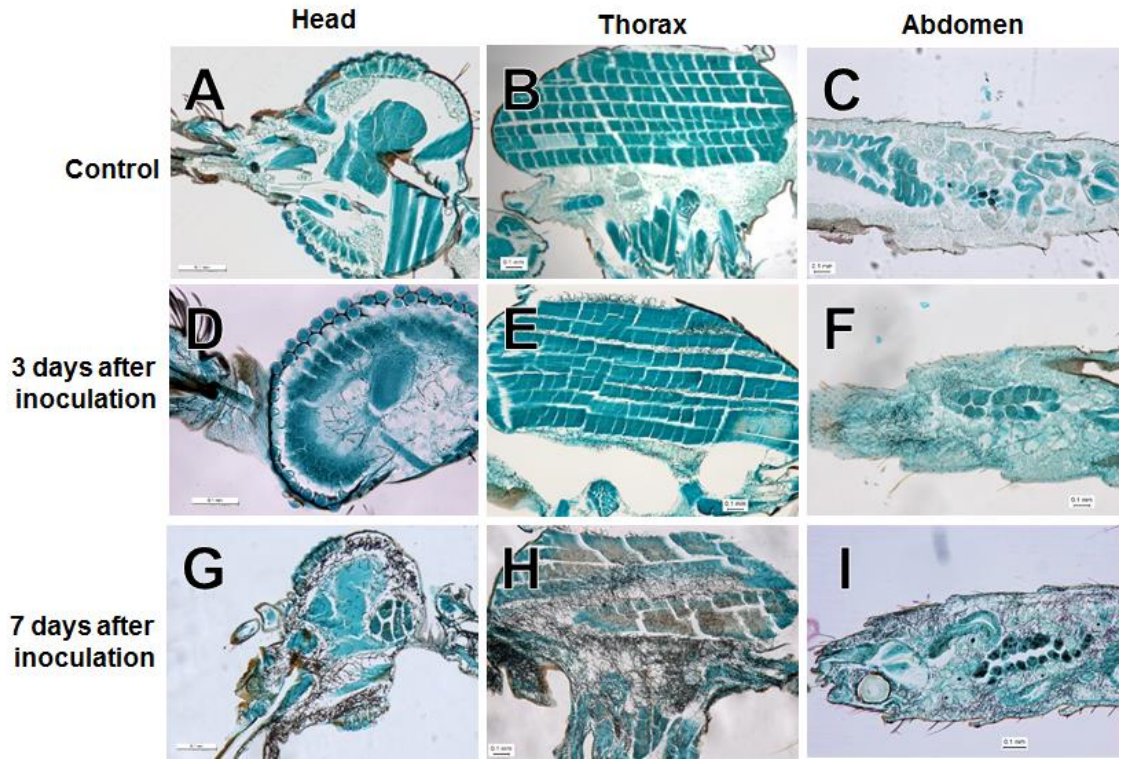

Figure 2. Images of uninfected and infected mosquito body parts. (A-C) *An. stephensi* female adult without fungal infection in control. (D-F) Fungal infection to *An. stephensi* female adult at early stage of infection (3 days after inoculation). (G-I) Fungal infection to *An. stephensi* female adult at later stage of infection (7 days after inoculation). (A, D and G) Head part. (B, E and H) Thorax part. (C, F and I) Abdomen part. Mosquito tissues and organs were stained with fast green, and fungal propagules were specifically stained with black by Grocott stain.

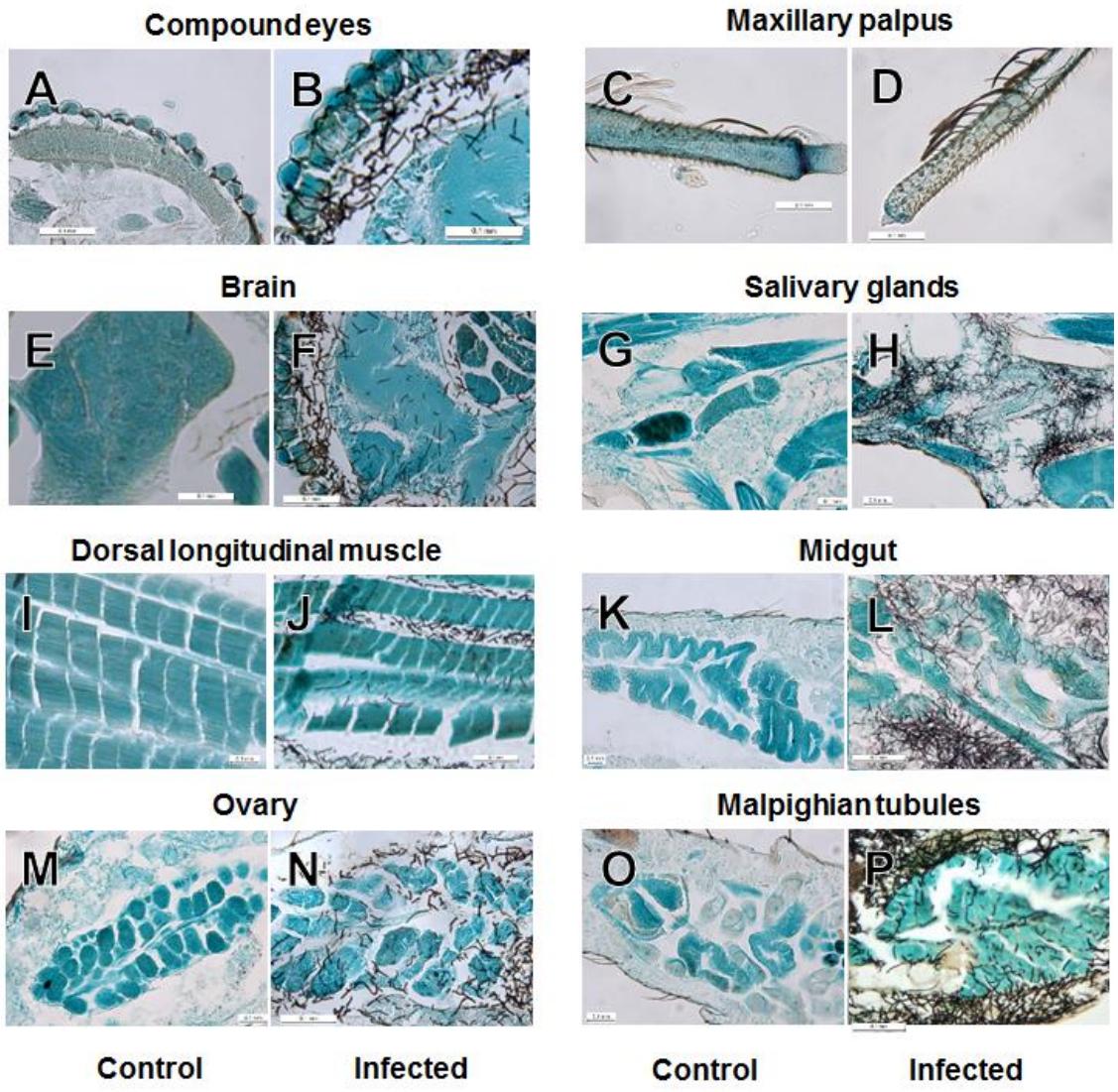

Fig.2-3. Images of uninfected and infected mosquito body parts. (A-C) *An. stephensi* female adult without fungal infection in control. (D-F) Fungal infection to *An. stephensi* female adult at early stage of infection (3 days after inoculation). (G-I) Fungal infection to *An. stephensi* female adult at later stage of infection (7 days after inoculation). (A, D and G) Head part. (B, E and H) Thorax part. (C, F and I) Abdomen part. Mosquito tissues and organs were stained with fast green, and fungal propagules were specifically stained with black by Grocott stain.

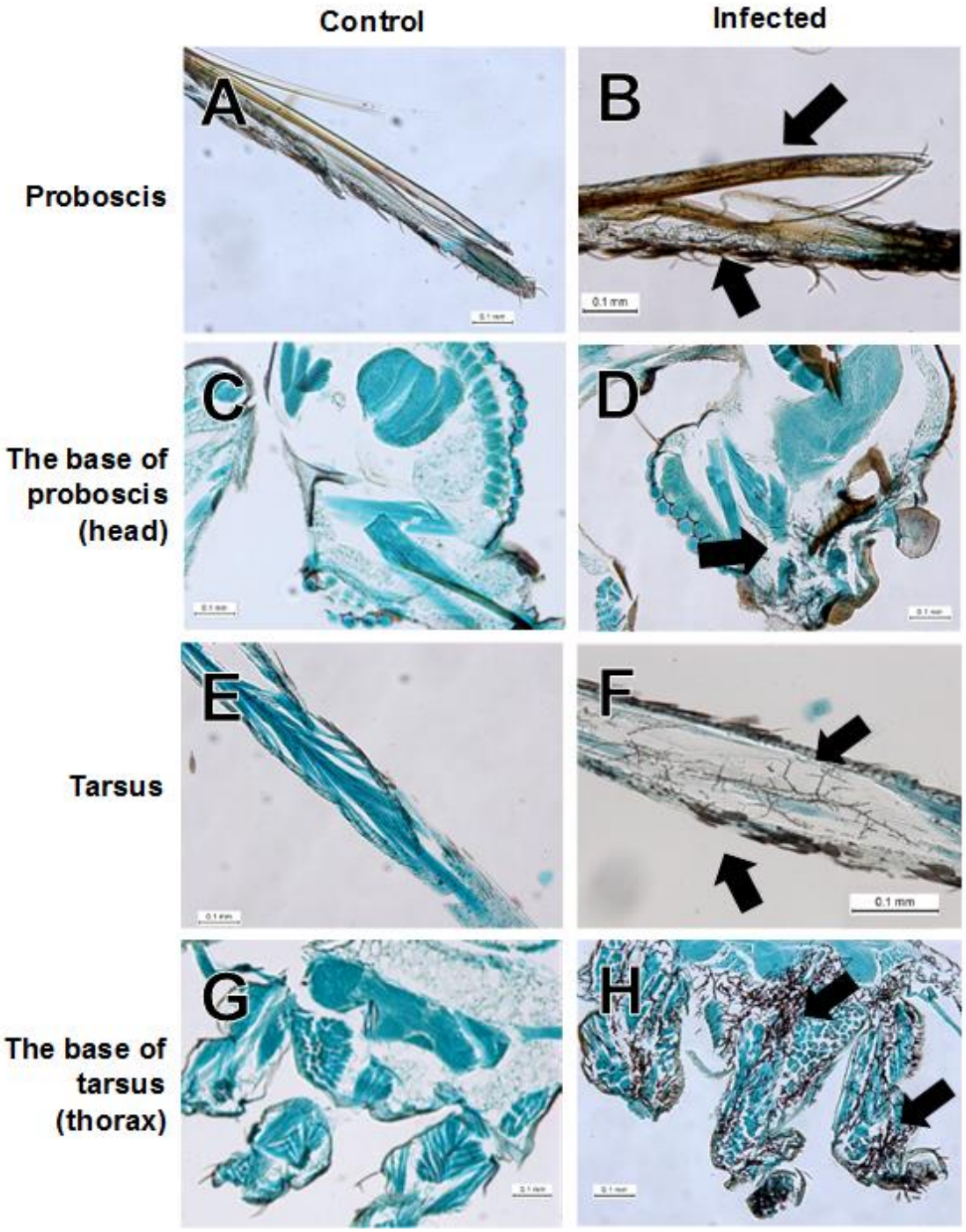

Fig. 2-4. Images of fungal invasion to various tissues and organs of *An. stephensi* female adult. (A, C, E, G, I, K, M and O) Uninfected tissues and organs in control. (B, D, F, H, J, L, N and P) Infected tissues and organs. (A and B) Compound eyes. (C and D) Maxillary palpus. (E and F) Brain. (G and H) Salivary glands. (I and J) Dorsal longitudinal muscle. (K and L) Midgut. (M and N) Ovary. (O and P) Malpighian tubules.

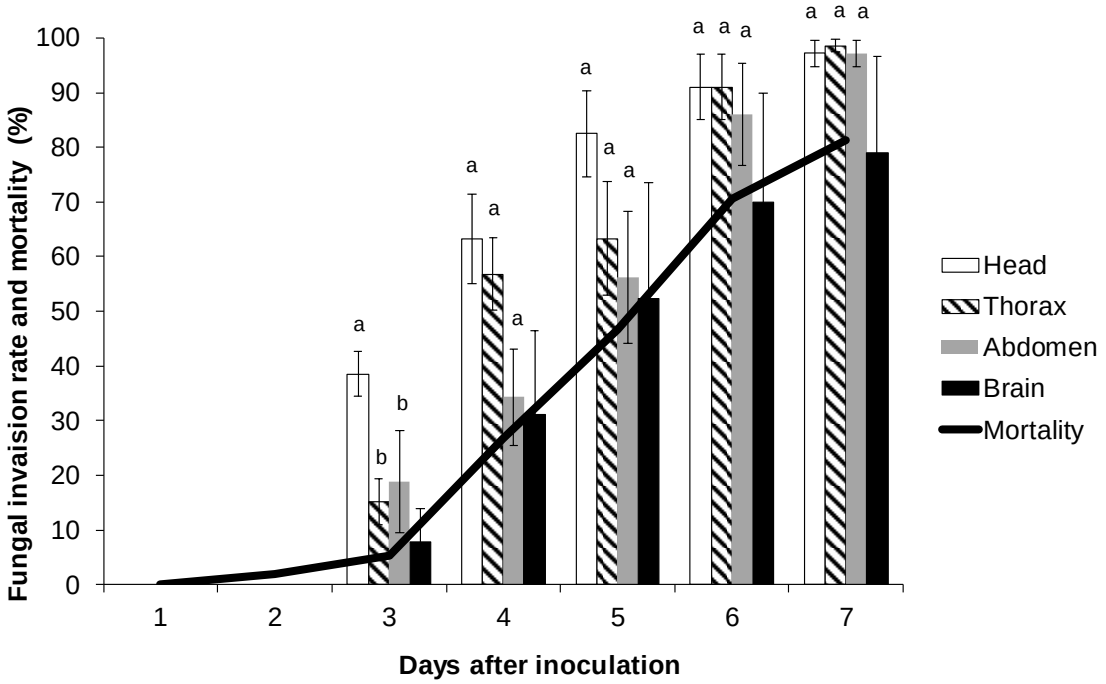

Fig. 2-5. Mean fungal invasion rate to each mosquito body parts and the mortality. Fungal invasion to each mosquito body parts were evaluated at 1 to 7 days after inoculation and the mortality of *An. stephensi* (%) was monitored until 7 days after inoculation. For each days after inoculation, different letters indicate significant differences among body parts (n=30, $p < 0.05$, Tukey's honest significant difference test).

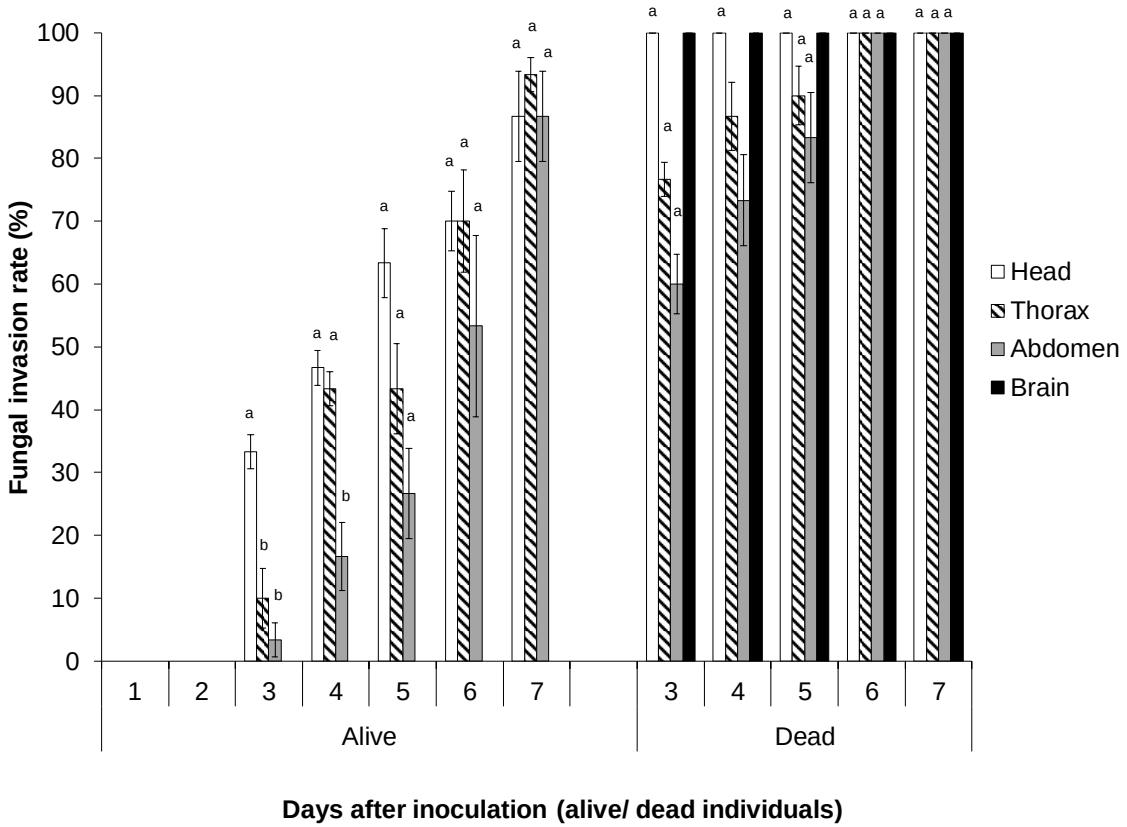

Fig. 2-6. Mean fungal invasion rate to each body parts on alive and dead mosquito. The infected alive and dead individuals were divided, and the fungal invasion rates of each parts were evaluated. The infected alive individuals were evaluated at 1 to 7 days after inoculation, and the infected dead individuals were evaluated at 3 to 7 days after inoculation because dead individuals were not detected at 1 to 2 days after inoculation. For each days after inoculation, different letters indicate significant differences among body parts (n=30, $p < 0.05$, Tukey's honest significant difference test).

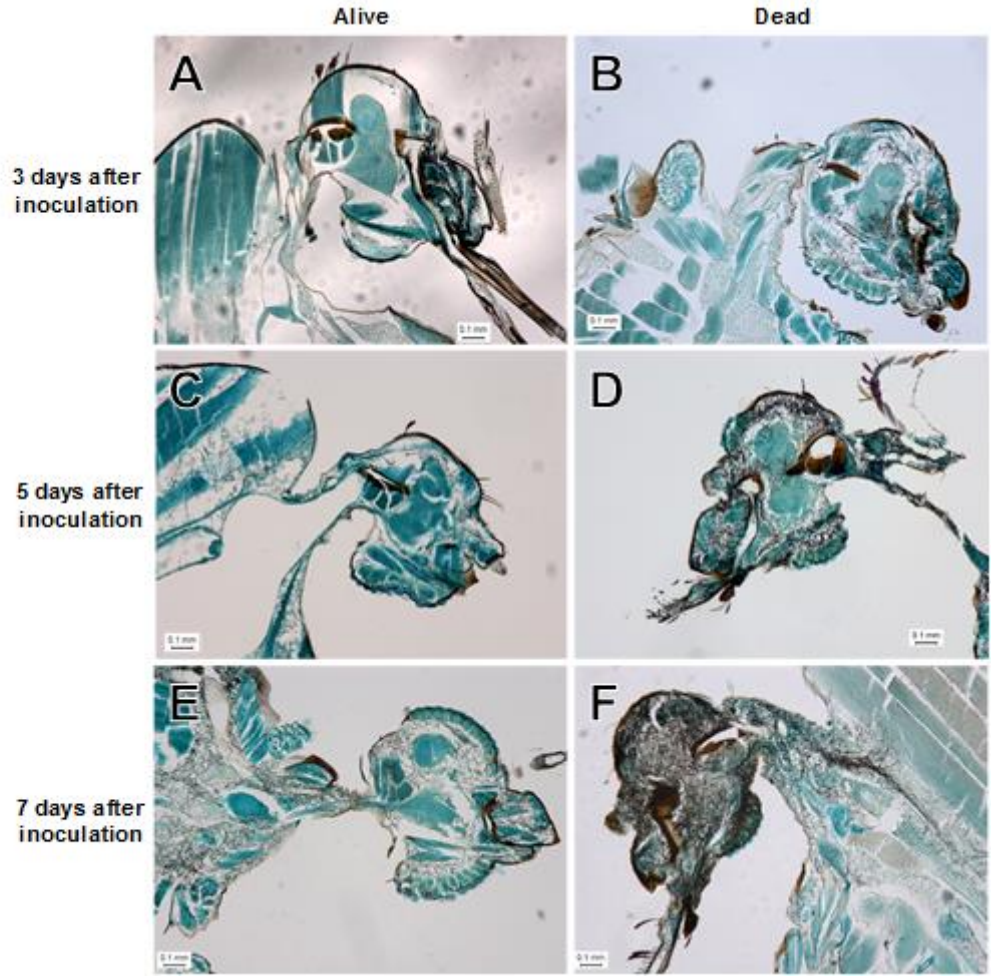

Fig. 2-7. Comparing fungal invasion to the head of infected alive and dead *An. stephensi*. (A, C and E) Uninfected brain in alive individuals. (B, D and F) Infected brain in dead individuals. (A and B) Infected head part at 3 days after inoculation. (C and D) Infected head part at 5 days after inoculation. (E and F) Infected head part at 7 days after inoculation.

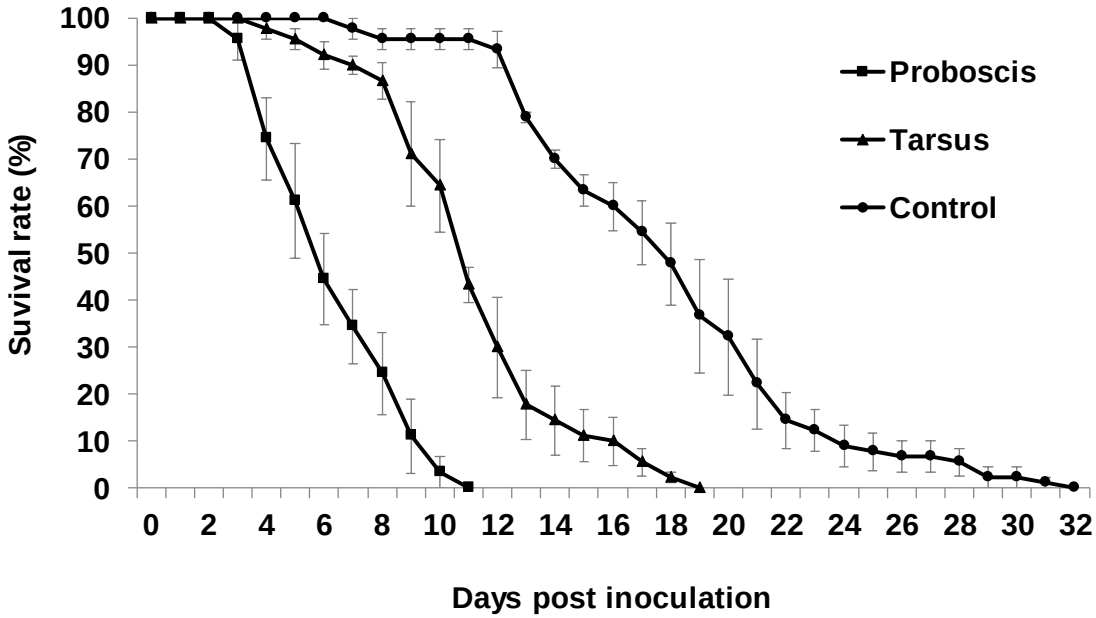

Fig. 2-8. Comparison of the survival rate of different inoculation route from the proboscis and tarsus. The survival rate of fungal inoculations to the proboscis and tarsus were evaluated until all individuals were dead. As for the survival rates, there are significant differences among the proboscis inoculation, the tarsus inoculation and control (n=90, $p < 0.01$, log rank test).

Table 2-1. Correlation coefficient between the fungal invasion rate to each part and the mortality. The estimate represents the correlation coefficient between the fungal invasion rate to each part (head, thorax, abdomen and brain) and the mortality. Estimates, r, t, df and p-values (n=30, Person's product-moment correlation).

	r	t	df	p-value
Head	0.8550571	7.1878	19	7.912E-07
Thorax	0.8909442	8.5519	19	6.136E-08
Abdomen	0.9116348	9.6684	19	9.035E-09
Brain	0.9889223	29.041	19	< 2.2e-16

II-5. Summary

Entomopathogenic fungi are known to control vector mosquitoes. It is important to understand the infection dynamics of entomopathogenic fungi for the effective control of insect pests. We investigated the infection dynamics between *B. bassiana s.l.* 60-2 and *Anopheles stephensi* by tarsus topical inoculation method, and compared the mortality and extent of infection to determine the lethal factor. Fungal development was observed from not only tarsus but also proboscis by this inoculation method, and early death of mosquitoes occurred only upon infecting through the proboscis route. Fungal hyphae invaded almost all the tissues and organs before or after the death of host and fungal invasion to brain correlated highly with the mortality. Although all the living individuals were not infected with fungus in their brain, fungal infection to brain was observed in all the dead individuals. Our results suggest that fungal invasion by entomopathogenic fungi to brain and the proboscis route of infection are important to control vector mosquitoes.

Chapter III

An entomopathogenic fungus strain of *Beauveria bassiana* inactivate disease transmission and reproductive capability against *Anopheles stephensi*

III-1. Introductions

In Chapter II, fungal infection was occurred on proboscis and legs of mosquito by tarsus topical inoculation method, and fungal invasion speed to the head part was faster than other parts. Female mosquitoes search target hosts using multiple sensory inputs, including heat, visual cues and odors, and they have such sensory organs on their head. Additionally, fungal invasion was observed from various organs before host death. Probably, fungal invasion to each organs and tissues might affect to mosquito behavior and/or their reproduction.

Actually, some researches showed influences of entomopathogenic fungi on host behavior; *M. anisopliae* infection reduced blood feeding and fecundity against *An. gambiae* (Scholte et al., 2006), and *B. bassiana* showed not only lethal effect but also sub-lethal effects such as reduction of response to the frequency of glucose and blood feeding against *An. stephensi* (Blanford et al., 2011). Host searching behavior to 1-octen-3-ol and responsiveness of olfactory receptor neuron were reduced by infection of *B. bassiana* and *Metarhizium acridum* (George et al., 2011).

This study was focused on a behavioral alteration of the mosquito by *B. bassiana s.l.* 60-2 infection, and considered the interaction between the behavioral alteration (in this Chapter) and fungal invasion to each parts (shown in Chapter II). In previous

researches, only lethal effect or one of sub-lethal effect has been evaluated. In general, mosquitoes recognize hosts by using some kind of attractants (e.g. heat, color and odor), and then they take a blood meal on hosts (Kusakabe and Ikeshoji, 1990). After blood feeding, their follicles are grown in the ovary, and mosquitoes produce next generation (Klowden, 1997). In odor to understand the total effect of entomopathogenic fungi, it is necessary to evaluate influence on all stages (e.g. host searching, blood feeding and egg production) of infected mosquitoes. In this study, sub-lethal effects of entomopathogenic fungus *B. bassiana s.l.* 60-2 (e.g. influence to host searching behavior to three kind of attractant, frequency and amount of blood feeding, and reproductive capability) were evaluated comprehensively. Firstly, host searching behaviors to heat and color were evaluated by using automatic recording device, and host searching behavior to odor was evaluated by using Y-tube olfactometer. Subsequently, frequency and amount of blood feeding, and then egg production were evaluated. Finally, follicle development and egg hatching were evaluated.

Since it takes 10-14 days for the malaria parasite to develop within a mosquito following a blood feed, the life-shortening effects of fungal infection can dramatically reduce malaria transmission potential (Blanford et al, 2005, Scholte et al., 2005, Read and Thomas, 2009 and Hancock et al., 2009). In addition, if such kind of behaviors (e.g. host

searching behavior and blood feeding behavior) will be alter, these fungal effects also might inhibit disease transmission. In brief, it does not necessarily matter whether the mosquito is alive or dead. The results will provide true control effect of entomopathogenic fungi including potential for reduction of malaria transmission and the vector's propagation.

III-2. Materials & Methods

III-2.1. Fungus

B. bassiana s.l. 60-2 was isolated from wild mosquitoes in Japan and showed highest virulence and infectivity against *An. stephensi* in (Chapter I). The fungal isolate was cultured on a 90 mm potato dextrose agar plate at 24 °C in the dark for 10-15 days. The germination rate of the fungal isolate was more than 99%.

III-2.2. Mosquitoes

The larvae of *An. stephensi* were reared for approximately 10 days in plastic trays (20.5 × 26.5 × 4.5 cm). The larvae were fed Hikari Economy® (KYORIN CO., LTD., Japan) fish food daily. The pupae were collected daily and transferred to mesh cages (27 × 27 ×

27 cm). Adults emerged were fed on 10 % (w/v) sucrose/water solution ad libitum. At all developmental stages, mosquitoes were kept in an incubator at 27 °C and 16:8 h light: dark. For the bioassay, 4-6 days old adult female non blood feeding mosquitoes were transferred from the adult cage to an acrylic bottle (2.7 cm in diameter × 12 cm in height) using a mouth aspirator.

III-2.3. Fungal inoculation method

The spore concentration was adjusted to 1.3×10^7 conidia ml^{-1} in 0.05 % Tween 20 solution by a hemacytometer. One milliliter of this suspension was pipetted evenly over a 90 mm diam filter paper, resulting in conidial densities of 2.0×10^{10} conidia/m^2. As control, 1 milliliter of 0.05% Tween 20 solution was pipetted evenly over the filter paper. This filter paper was then placed in the lid of a 90-mm-diameter Petri dish.

Mosquitoes were transferred from the adult cage to an acrylic bottle and were anesthetized in cold condition (at 5-10 °C) because carbon dioxide gas affect to mosquito behavior. The mosquitoes were transferred to an exposure Petri dish that was prepared as described in the previous paragraph and were placed in direct contact with the fungal inoculum on the filter paper. After recovering from the anesthesia, the mosquitoes were allowed to walk on the inoculum for 30 min. After exposure, the mosquitoes were

transferred to bottom-meshed assay tubes (8.5 cm in diameter and 9.5 cm in height), which were covered with nylon socks with the toe part cut off. This inoculation method referenced by the following literature (Farenhorst and Knols, 2010). The mosquitoes were kept at 27 ± 1 °C, 80 ± 4 % relative humidity and 16:8 h light: dark. The mosquitoes were fed on 10 % (w/v) sucrose/water solution ad libitum.

III-2.4. Automatic recording device for quantifying host searching behaviors to the heat and color

This recording device was designed by Maekawa et al. (2011) and modified for the present study. The summary of this recording device was shown in (Fig.3-1). This device is able to simultaneously monitor and quantify three independent mosquito behaviors (touch-down on target, sugar-feeding and mock) for free-fry mosquito. At first, some kind of attractants were set inside of the target box in this device. Then mosquitoes were attracted to the attractant, and touch down to the target box. During this period, mosquito block infrared laser, and this information was automatically counted by PC. The recording device was composed basically of three infrared laser sensors (LV-H300, Keyence, Osaka, Japan), amplifiers (LV-51M, Keyence, Osaka, Japan), a programmable controller unit (KV-3000, Keyence, Osaka, Japan), power unit (KV-U7, Keyence, Osaka, Japan) and

monitoring software (KV-H4W, Keyence, Osaka, Japan). The infrared laser sensor was composed of a laser releaser and acceptor kept approximately 30 cm apart. These 3 sensors were placed in parallel at the bottom of a large nylon mesh and metal frame cage (90 cm × 55 cm × 50 cm) set in an incubator (LPH-350SP, Nippon Medical & Chemical Instruments Co., Ltd., Osaka, Japan) maintained at 27°C, 16 h: 8 h = L: D photoperiod (Light period: 6:00-20:00; Dark period: 22:00-6:00) and 80% RH. In order to measure three kinds of mosquito behaviors (host searching, sugar feeding, and control), some kind of attractant (e.g. heat and color), 10% sucrose solution, and Mock target (a white paper), respectively, were placed at the center of each sensor.

In odor to evaluate the host searching behavior to the heat, the heat target (a white paper with heat, 40°C), 10% sucrose solution and Mock target were placed in the target box. To heat a white paper, the surface of Peltier plate was covered with a white paper, and the temperature of the Peltier plates (VPE20-30S, VICS, Koriyama, Japan) was set at 40°C. To activate mosquito behavior, CO_2 release (2 s at 15 min intervals) from a nozzle at the top of the cage was controlled by a solenoid valve (FSD-0408C, Flon Industry, Tokyo, Japan) equipped with intermittent timer FT-022 (FT-011, Flon Industry, Tokyo, Japan). The CO_2 density inside the devise was set so that 3500-4000ppm become the peak. After fungal inoculation immediately, 50 female adult mosquitoes were released

in the recording devise, and the numbers of the touchdown to each stage were recorded for 10 days. Mortality was monitored daily for 10 days and the cadavers were removed from this recording device. Only alive individuals were evaluated their behaviors, and the average of touchdown per alive mosquito was calculated. As a control, non-infected mosquitoes were used in this experiment. This experiment was started at 18:00, and conducted in triplicates.

In odor to evaluate the host searching behavior to the black color, the black target (a black paper without heat), 10% sucrose solution and Mock target were placed in the target box. The following experiment procedures are same as the above section.

III-2.5. Y-tube olfactometer for quantifying the host searching behavior to the odor

To attract the mosquitoes, yeast-generated CO_2 and ethyl alcohol as odor source were generated by 500 ml of water, 50 g of sucrose and 1.4 g of dry-yeast in a 2 L plastic bottle. This method could generate 32.4 ml/min of CO_2 (1600-1700 ppm) for at least 27 h under 25-27°C. The efficacy of yeast-generated CO_2 as attractant for 6 mosquito species were significant (Saitoh et al., 2004 and Silver, 2008).

The Y-tube olfactometer was shown in (Figure 3-2). This Y-tube olfactometer was passed air through the arms by an air pomp, creating an airflow of 3.0ml/s. Airflow

was regulated with flow meters, and air was bubbled through a water or the odor souse with a water to provide humidity. The temperature in the bioassay room was maintained at 24-27°C and 16 h: 8 h = L: D photoperiod (Light period: 6:00-22:00; Dark period: 22:00-6:00), and the relative humidity control of inside of Y-tube olfactometer was kept over 90% RH. This experiment was started at 18:00.

As preliminary examination, non-infected mosquitoes were released in the Y-tube olfactometer. To decide test duration, the number of mosquitoes in each arm (air or odor) were counted after 1, 3, 4, 6, 12, and 24h from the start of this experiment. This experiment was conducted in 4 replicates.

On 1, 2, 3, 4, 5, 6 and 7 days after inoculation, 30 alive infected mosquitoes were collected. Each infected 30 mosquitoes were released in Y-tube olfactometer. Attraction to the odors was evaluated by counting the number of mosquitoes in air arm and odor arm at 6 h after mosquito release. As a control, non-infected mosquitoes which is same day-old with each infected mosquitoes (at 1, 2, 3, 4, 5, 6 and 7 days after inoculation, respectively) were used. This experiment was conducted in 10 replicates.

III-2.6. Effect of fungal infection on blood feeding and egg production

In this experiments, effects of fungal infection on the frequency and amount of blood

feeding, and the number of egg production were evaluated. In total of 210 non blood feeding mosquitoes (seven treatments; 30 mosquitoes per treatment) were inoculated by the tarsus topical inoculation, and then each 30 mosquitoes were transferred to a bottom-meshed assay tubes, which were covered with nylon socks with the toe part cut off. Mortality was monitored daily for 10 days, and the cadavers were removed from this bottom-meshed assay tubes.

On 1, 2, 3, 4, 5, 6 and 7 days after inoculation, each alive mosquitoes were fed on defibrinated blood of a horse by membrane feeding (40°C) in the bottom-meshed assay tubes for 15 min, and then the rate of blood feeding were evaluated (visual observation). Only mosquitoes which had taken a blood meal were used for experiment of the amount of blood feeding. The amount of blood feeding was calculated from a difference of blood weight of before and after blood feeding.

Subsequently, only alive mosquitoes which had taken a blood meal (visual observation) were used for the experiment of egg production. The mosquitoes were transferred to another bottom-meshed assay tubes with a cup for laying eggs in 2 days after blood feeding. After 3 days from a blood meal, the number of egg production was collected and counted in 3 days after blood feeding. The average of egg production per alive mosquito was calculated. This experiment was conducted in 5 replicates.

III-2.7. Effect of fungal infection on follicle development and hatching rate

In total about 900 mosquitoes (30 treatments: 30 mosquitoes per treatment) were inoculated by the tarsus topical inoculation, and then each 30 mosquitoes were transferred to the bottom-meshed assay tubes and reared until use. On 1, 2, 3, 4, 5, 6 and 7 days after inoculation, each 30 alive mosquitoes were collected and transferred to another bottom-meshed assay tubes. The 30 mosquitoes were fed on defibrinated blood of a horse by membrane feeding, and then only mosquitoes which had taken a blood meal (visual observation) were used in this experiment. After 48h from a blood meal, each 10 mosquitoes were selected randomly, and a follicle in the abdomen were dissected by using forceps under light microscope (Leica: DMI 3000B) at 10× magnification. To evaluate the follicle development, 100 follicles were collected randomly, and the shape and size of follicle was evaluated.

Subsequently, after 48h from a blood feeding, the other 10 mosquitoes which had taken a blood meal were selected randomly, and transfer to another bottom-meshed assay tubes with a cup for laying eggs. After 72h from a blood meal, the mosquitoes were laid eggs in the cage, and the 100 eggs were collected randomly and transferred to a plastic cup. After 24h from laying eggs, the hatching rate was evaluated. As a control, non-infected mosquitoes which is same day-old with infected mosquitoes at 7 days after

inoculation were used. This experiment was conducted in triplicates.

III-2.8. Statistical analysis

In the assay of automatic recording device, the number of the total touchdown (counts/ day) and the number of the touchdown per alive mosquito (counts/ day) for each attractant (heat or color, 10% sucrose solution, and a white paper) on each day after the inoculation were analyzed using ANOVA. When the Kruskal-Wallis test was significant at $p < 0.01$, the treatment means were compared using Steel-Dwass test *(*$p < 0.05$). In other experiments, the rate of attractant to the odor, blood feeding, egg production and follicle development, the rate of attractant to odor, the rate of blood feeding, the amount of blood feeding, the number of total egg production of alive mosquitoes, the average of egg production per alive mosquito, the rate of follicle development, and hatching rate were analyzed using ANOVA. When the F test was significant at $P < 0.01$, the treatment means were compared using Tukey's Honest Significant Difference test *(*$p < 0.05$).

III-3. Results

III-3.1. Effect of fungal infection on host searching behaviors to the heat and black

color

In the experiment by using automatic recording device, mosquitoes constantly touch downed to the heat target, 10% sucrose solution and Mock target for 10 days in control plot. Mosquitoes tended to touch down to the heat target during the dark period compared with the light period (Fig. 3-3A). Especially, the number of touchdown to the heat target (counts / day) was significantly higher than other targets on any days ($p < 0.05$) (Fig. 3-3B). However, touchdown to the heat target was drastically decreased (especially during dark period) from 3 days after inoculation in fungal inoculation plot (Fig. 3-3C). The number of the touchdown (counts/ day) to the heat target was significantly decreased from 3days after inoculation in fungal inoculation plot compared with control plot ($p < 0.05$) (Fig. 3-3B, D). The mosquitoes constantly touch downed to 10% sucrose solution and the Mock target until 8 days after inoculation, and the number of the touchdown (counts/ day) to 10% sucrose solution and the Mock target was significantly decreased from 9 days after inoculation in fungal inoculation plot compared with control plot ($p < 0.05$) (Fig. 3-3B, D). In contrast to the number of touchdown to the heat target (Fig. 3-3A), the mosquitoes tended to touchdown to the black target during the light period compared with the dark period (Fig. 3-4A). In the experiment of host searching behavior to the black color, mosquitoes constantly touch downed to the black target, 10% sucrose solution, and

the mock target for 10 days in control plot (Fig. 3-4A, B). The number of touchdown to the black target (counts / day) was significantly higher than other targets on any days ($p < 0.05$) (Fig. 3-4B). On the other hand, the number of touchdown to the black target tended to decrease from 6 days after inoculation in fungal inoculation plot (Fig. 3-4C, D). The number of the touchdown (counts/ day) to the black target was significantly decreased from 9 days after inoculation in fungal inoculation plot compared with control plot ($p < 0.05$) (Fig. 3-4B, D). Also the number of the touchdown (counts/ day) to 10% sucrose solution and the mock target was significantly decreased from 9 days after inoculation in fungal inoculation plot compared with control plot ($p < 0.05$) (Fig. 3-4B, D).

III-3.2. Effect of fungal infection on host searching behaviors to odors

As the preliminary experiment, it was confirmed that yeast-generated CO_2 and ethyl alcohol as odor source attract mosquitoes in the Y-tube olfactometer. The rates of attraction to air side were 19, 21, 11, 6, 23, 11 and 12% in 1, 3, 4, 6, 12 and 24 h after mosquito release, respectively (Fig. 3-5A). On the other hand, the rates of attraction to the odor side were 10, 22, 43, 48, 48, 81% in 1, 3, 4, 6, 12 and 24 h after mosquito release, respectively, and it has tendency to increase the choice to odor side according to the length

of experiment time (Fig. 3-5A). Especially, the rate of attraction to the odor side was significantly higher than air side in 6 and 24 h after mosquito release ($p < 0.05$). Here, I accepted the experimental duration for 6 h after mosquito release, because the rate of attractant to the odor side was significantly higher than air side at 6 h after mosquito release, and the time was earlier than 24h after mosquito release.

In control plot, the rate of attraction to the odor side showed around 50% and it was significantly higher than air side at any days ($p < 0.05$) (Fig. 3-5B). In fungal inoculation plot, although the rate of attraction to the odor side showed significantly higher than air side until 2 days after inoculation ($p < 0.05$), there are no significant difference between the odors and air side from 3 days after inoculation until 7 days after inoculation (Fig. 3-5C). Furthermore, the rate of attraction to the odor side was significantly decreased from 2 days after inoculation in fungal inoculation plot compared with control plot ($p < 0.05$) (Fig. 3-5B, C).

III-3.3. Effect of fungal infection on blood feeding behavior and egg production

In control plot, most mosquitoes constantly fed blood at any days, and the rates of blood feeing were around 70-80%. In fungal inoculation plot, the rates of blood feeing were 77, 69, 71, 42, 35, 40, 49 and 39% at 0, 1, 2, 3, 4, 5, 6 and 7 days after inoculation, respectively,

and it was significantly decreased from 3 days after inoculation compared with control plot ($p < 0.05$) (Fig. 3-6A). The mosquito fed around 3mg of defibrinated blood of horse at any days in control plot. On the other hand, it showed 3.3, 3.6, 2.8, 2.2, 2.3, 1.9, 1.7 and 1.6 mg at 0, 1, 2, 3, 4, 5, 6 and 7 days after inoculation, respectively in fungal inoculation plot. The amount of blood feeding was significantly decreased from 3 days after inoculation in fungal inoculation plot compared with control plot ($p < 0.05$) (Fig. 3-6B).

In three days after the blood feeding, the mosquito produced around 30-50 eggs in control plot. In fungal inoculation plot, the numbers of egg production were 41, 22, 12, 13, 15, 8, 8 and 9 eggs in 3, 4, 5, 6, 7, 8, 9 and 10 days after inoculation, and the number of egg production was significantly decreased from 3 days after inoculation compared with control plot ($p < 0.05$) (Fig. 3-6C).

III-3.4. Effect of fungal infection on follicle development and egg hatching

In 48 h after blood feeding, follicle development is shown in (Fig 3-7A). Follicle development were observed in non-infected mosquitoes which remained a blood in their midgut (Fig. 3-7B). However, follicle development was not observed in most infected mosquitoes which remained a blood in their midgut (Fig. 3-7C). In the control plot, the

rate of follicle development was 95%. In fungal inoculation plot, the rate of follicle development was significantly decreased from 2 days after inoculation compared with control plot ($p < 0.05$) (Fig. 3-7D).

In control plot, eggs hatched in 4 days after blood feeding. Although 81% eggs were hatched in control plot, the hatching rate showed lower value at any days after inoculation in fungal inoculation plot. The hatching rates were 70, 63, 60, 44, 23, 30 and 25% in 4, 5, 6, 7, 8, 9 and 10 days after inoculation. The hatching rate was significantly decreased from 6 days after inoculation in fungal inoculation plot compared with control plot ($p < 0.05$) (Fig. 3-7E).

III-4. Discussions

This study confirmed the property of *B. bassiana s.l.* 60-2 which showed highest virulence against *An. stephensi* in (Chapter I). As a results, *B. bassiana s.l.* 60-2 infection affected to various behavior and reproduction against *An. stephensi*. The findings from this research supports previous studies (e.g. George et al., 2011, Blanford et al., 2011 and Scholte et al., 2006). This is an unprecedented study that various sub-lethal effects were evaluated comprehensively by only one of the fungal isolate against vector mosquito (e.g.

influence to host searching, blood feeding, egg production and follicle development). Moreover, this is the first study that, to our knowledge, evaluates the host searching behavior to the heat and black color by fungal infection. Especially, the host searching behavior to the heat was drastically decreased on early stage infection (i.e. 3 days after inoculation). These findings might help for vector control to exploit entomopathogenic fungi as biological control agents.

Our results showed that host searching behavior to each attractant and frequency of blood feeding in *An. stephensi* were significantly decreased by *B. bassiana s.l.* 60-2 infection (Fig. 3-3-6). Many species of mosquitoes including *Anopheles* spp. utilize CO_2 as olfactory cues in host searching (Jones et al., 2007 and Lu et al., 2007). CO_2 stimulation synergizes responses to host odor and by itself induces take-off and sustained flight behaviors in host searching of *Anopheles* spp. (Gillies, 1980 and Dekker et al., 2001). George et al., (2011) demonstrated that the reductions of mosquito host searching behavior by fungal infection correlates with a decline in electrophysiological sensitivity of the olfactory organ (i.e. maxillary palps). Mosquitoes recognize CO_2 and heat as their hosts using various sensory organs such as antennae and maxillary palpus (Roth and Willis, 1952, Ismail, 1962 and Steward and Atwood, 1963). Recent study showed that also proboscis and leg related to recognition of CO_2 and heat (Maekawa et al., 2011). In

(Chapter II), it was revealed that *B. bassiana s.l.* 60-2 infection was occurred on proboscis and legs by tarsus inoculation method, and the fungal invasion was observed various tissues and organs including sensory organs before death of the mosquito. Probably, such an infection dynamics of *B. bassiana s.l.* 60-2 (i.e. mechanical disruption of sensory organs) might affect to the host searching and blood feeding behaviors. On the other hand, other studies of grasshopper and click beetle indicated that reduction of feeding by fungal infection might be attributed to mechanical disruption of tissues and organs, and in combination with the production of secondary metabolites (Thomas et al., 1997 and Zacharuk, 1971). It was known that *Beauveria* spp. produce several secondary metabolites (e.g. beauvericin, bassianolide, bassianin and tenellin) as toxic compounds (Mazet et al, 1994., Strasser et al. 2000 and Vey et al., 2001). For example, beauvericin which is one of the secondary metabolites produced by *B. bassiana*, has insectidal, antibiotic, cytotoxic and ionophoric properties (Zimmermann et al., 2007). According to Roberts (1981), some toxic effects have been noticed against bacteria, mosquito larvae, brine shrimp and adult houseflies. Also *B. bassiana s.l.* 60-2 might produce some kind of secondary metabolite, and it is possible that the metabolite affects to the mosquito's behaviors. In further study, we need identify the secondary metabolite of *B. bassiana s.l.* 60-2, and elucidate the influence of the secondary metabolite against *An. stephensi*.

As another sub-lethal effect of *B. bassiana s.l.* 60-2 infection against *An. stephensi*, the blood feeding, egg production and also follicle development were significantly decreased (Fig. 3-6-7). Although other studies demonstrated that fungal infection reduced blood feeding and egg production in vector mosquitoes (e.g. Blanford et al., 2005, 2011, Howard et al., 2010 and Scholte et al., 2005), the mechanisms involved reduction of blood feeding are unclear. It has been suggested that reductions of feeding are due to resource competition within the host and/or mechanical damage of host tissue by the fungal invasion and proliferation in the study of *Ocinara varians* and *Chilo partellus* (Hussain et al., 2009 and Tefera et al., 2004). Entomopathogenic fungi appear to go through a period of very low replication after infection followed by rapid increase in biomass just prior to host death (Bell et al., 2009 and Anderson et al., 2011). Blanford et al., (2012) concluded that such growth patterns could explain the reductions in feeding, especially for the higher virulence isolates. *B. bassiana s.l.* 60-2 showed highest virulence in (Chapter I), and higher virulence compared with other studies (Achonduh and Tondje, 2008, Howard et al., 2010 and Scholte et al., 2006). It is possible that *B. bassiana s.l.* 60-2 also shows similar growth patterns, and this phenomenon might result in reduction of blood feeding in *An. stephensi*. As other possibility, *M. anisopliae* is known to produce their own enzymes for converting the stored sugars (e.g. trehalose) to glucose in insect

host (Xia et al., 2002). In phytophagous insects one of the feedback mechanisms implicated in decreasing motivation to feed involves a concentration gradient of glucose between the gut and hemolymph (Chapman, 1998). Probably, fungal-induced changes in key nutrient gradients such as glucose could also play a role here (Clements, 1999). Egg production was also decreased by the fungal infection in this study. Egg production in insects is affected by the amount of protein in the diet (Engelmann, 1984 and Klowden and Briegel, 1994), and it was known that the amount of blood feeding and egg production have strong positive correlation in seven species of *Anopheles* mosquitoes (Phasomkusolsil et al., 2015). It was suggested that the reduction of egg production caused by the reduction of the amount of blood feeding. Moreover, several study suggested that secondary metabolites are produced by the fungus act on insect tissues including midgut, and it might be responsible for the loss of appetite (e.g. Samuels et al., 1988, Thomas and Read, 1997 and Zacharuk, 1971). The mechanical block and/or production of secondary metabolites in the midgut by fungal infection might cause indigestion of a blood in the mosquito. Fungal invasion to the midgut was observed in alive infected mosquitoes in (Chapter II). The indigestion of a blood in the midgut by the fungal infection might indirectly result in reduction of egg production, follicle development and hatching because follicle development was not observed in infected

mosquitoes which remained a blood in their midgut. We need more information about mechanisms of sub-lethal effects (e.g. reduction of blood feeding and egg production), and it might be able to apply vector control.

In conclusion, it was demonstrated that *B. bassiana s.l.* 60-2 has various sub-lethal effects (i.e. reduction of host searching behavior to the heat, color and odor, blood feeding, egg production, follicle development and egg hatching) against *An. stephensi* in this study. In (Chapter I), *B. bassiana s.l.* 60-2 killed most of the *An. stephensi* individuals within 10 days, which is shorter than the EIP of the malaria parasite. It is important to incapacitate mosquitoes within this period to decrease the risk of transmitting malaria (Billingsley, 2010). Additionally, the sub-lethal effects of *B. bassiana s.l.* 60-2 are likely to further reduce the risk of a mosquito transmitting malaria, and also it could inhibit propagation of *An. stephensi* on all stages. The effect to propagation (i.e. reduction of mosquito population) might result in reduce malaria transmission indirectly. Such effects might able to apply for not only the infectious disease vector but also other vectors (e.g. agricultural insect pests). In agriculture, sub-lethal effects of fungal infection in addition to the mortality might act an important role in reducing the damage caused by target insects (Thomas et al., 1997). Therefore, these findings should contribute in establishing new biocontrol approaches against various vectors.

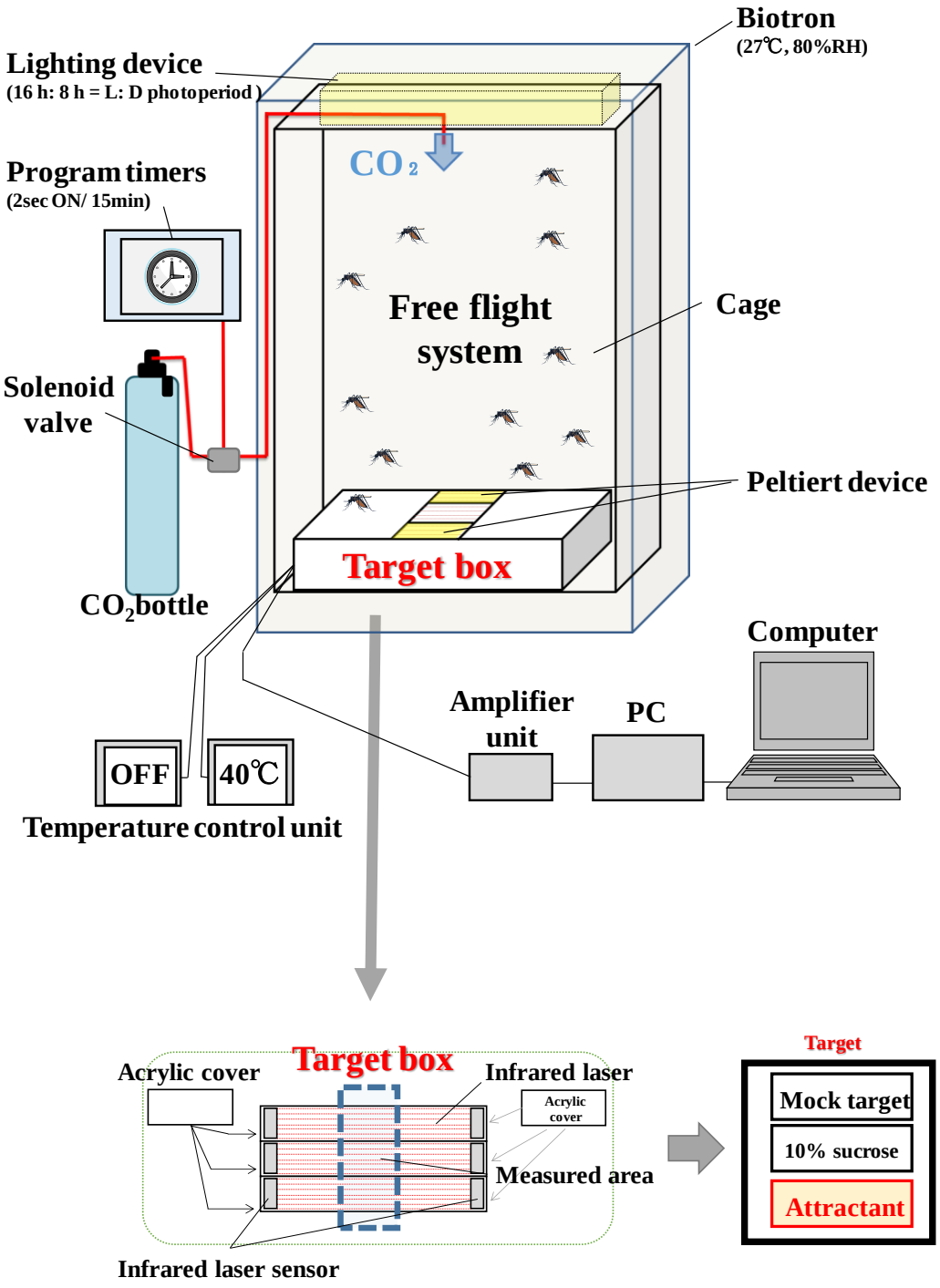

Fig. 3-1. Over view of automatic recording device to quantify host searching behavior to each attractant (see Materials and Methods).

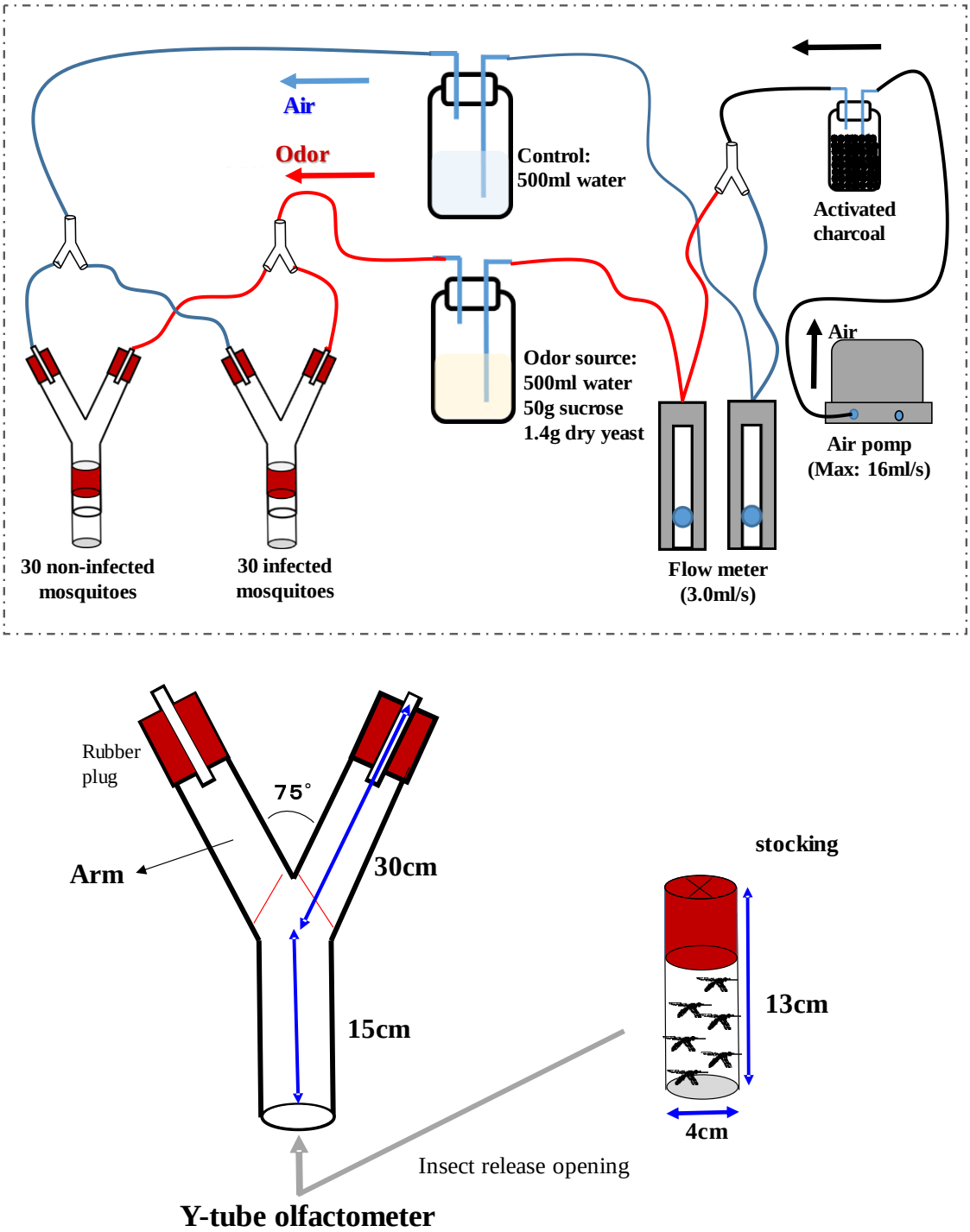

Fig. 3-2. Over view of Y-tube olfactometer to quantify host searching behavior to the odor (i.e. yeast-generated CO_2 and ethyl alcohol).

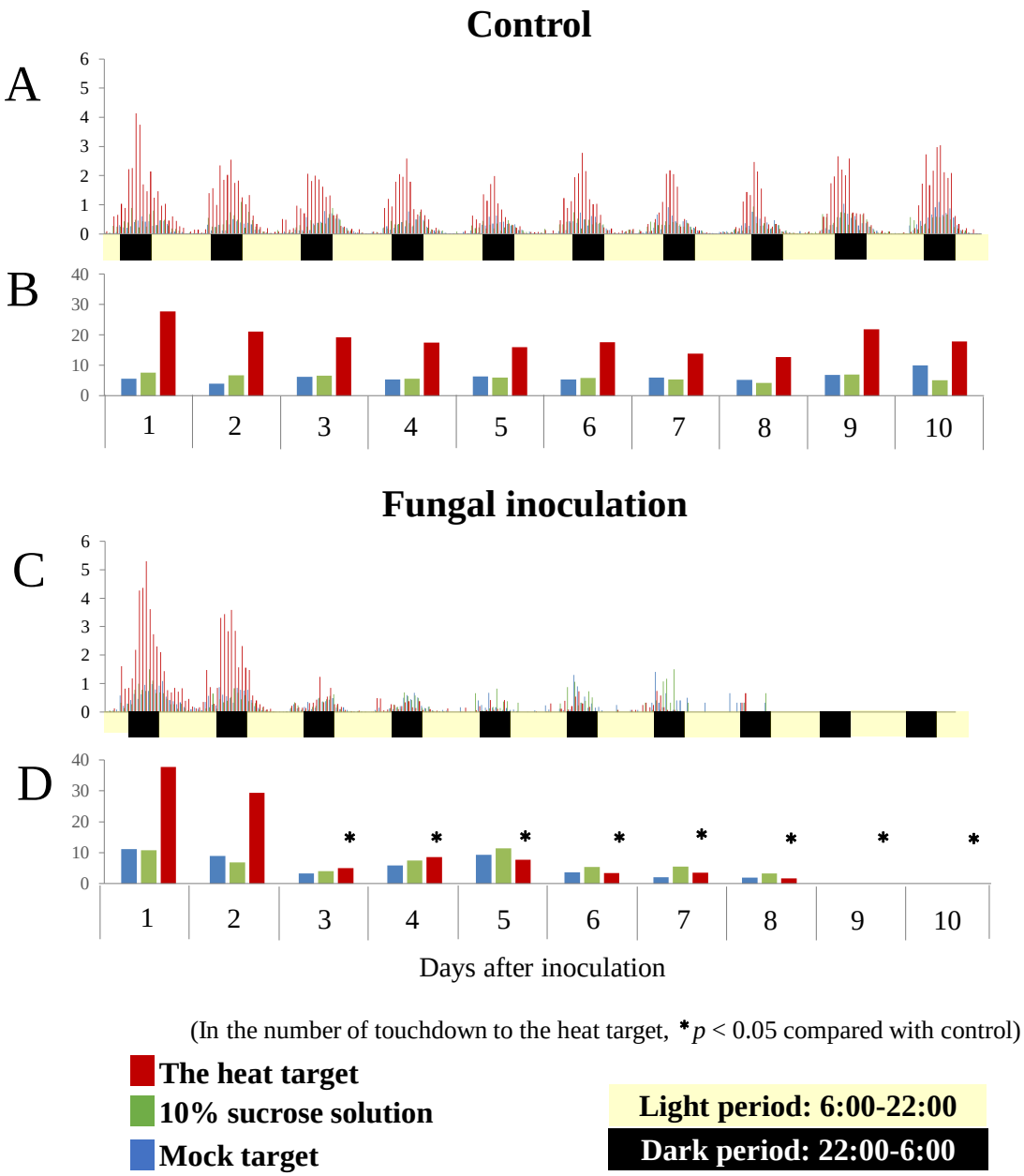

Fig. 3-3. Effect of fungal infection on host searching behaviors to the heat target in automatic recording device. (A, B) The number of touchdown in control plot. (C, D) The number touchdown in fungal inoculation plot. (A, C) The number of the touchdown per alive mosquito (counts/ hour). (B, D) The number of the touchdown per alive mosquito (counts/ day). The behavioral assay was performed for 10days under each condition with the heat target (red), 10% sucrose solution (green), and Mock target (blue), respectively. CO2 is delivered intermittently (2 s every 15 min) during 10 days. Under crossbar indicates light (yellow)-dark (black) timer controlled conditions (6:00 ON; 22:00 OFF).

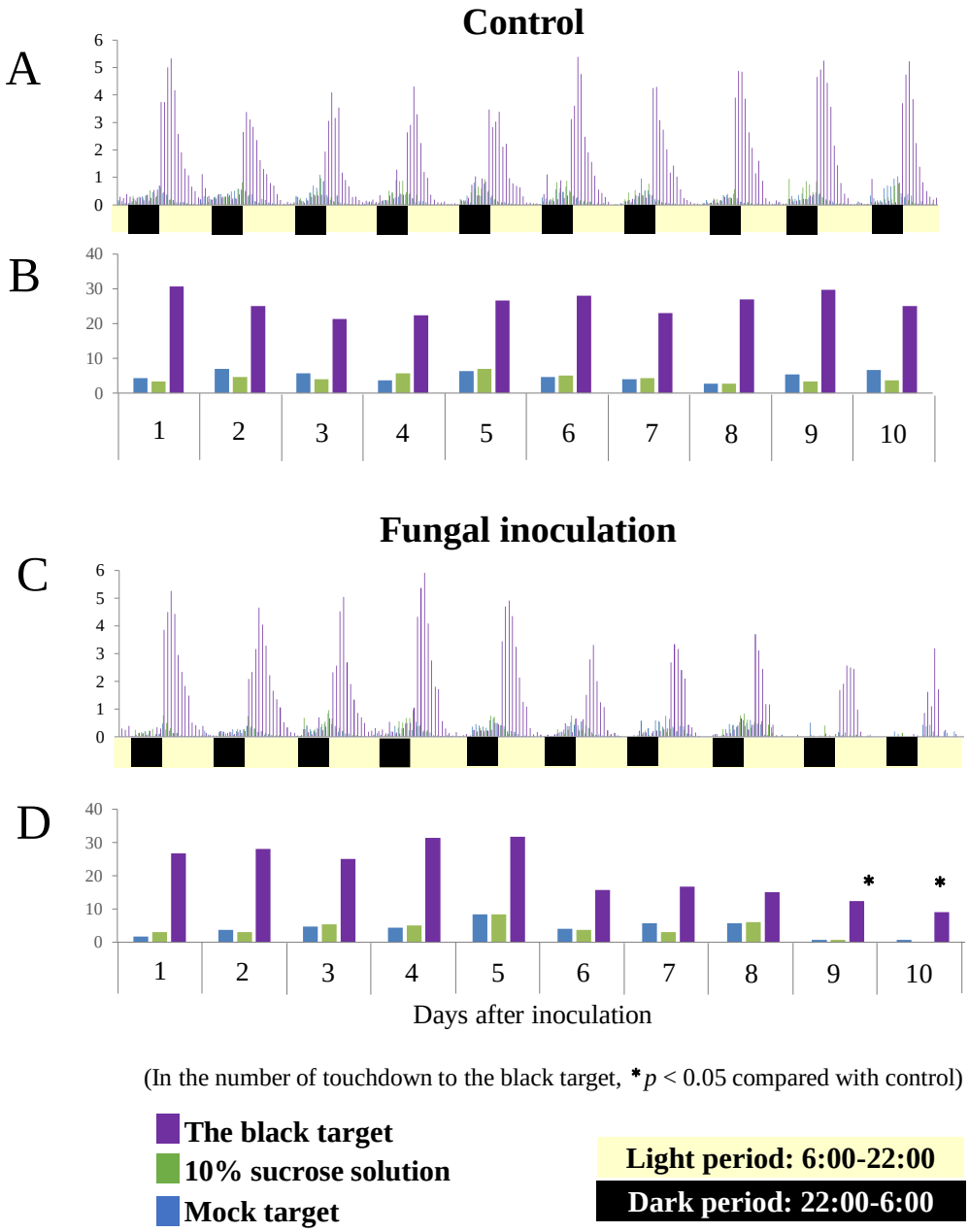

Fig. 3-4. Effect of fungal infection on host searching behaviors to the black target in automatic recording device. (A, B) The number of touchdown in control plot. (C, D) The number touchdown in fungal inoculation plot. (A, C) The number of the touchdown per alive mosquito (counts/ hour). (B, D) The number of the touchdown per alive mosquito (counts/ day). The behavioral assay was performed for 10days under each condition with the black target (purple), 10% sucrose solution (green), and Mock target (blue), respectively. CO_2 is delivered intermittently (2 s every 15 min) during 10 days. Under crossbar indicates light (yellow)-dark (black) timer controlled conditions (6:00 ON; 22:00 OFF).

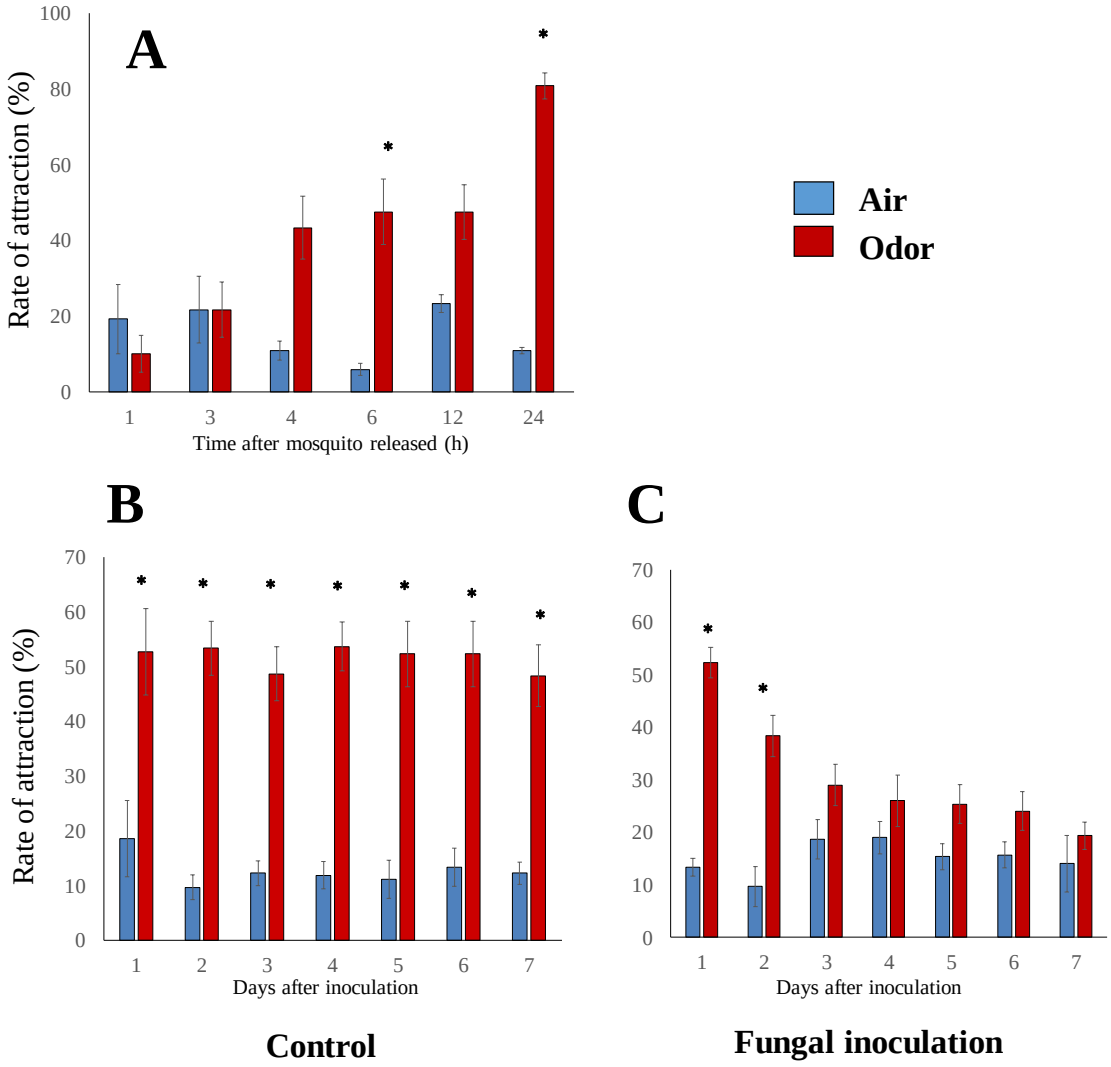

Fig. 3-5. Effect of fungal infection on host searching behavior to the odor (yeast-generated CO_2 and ethyl alcohol) in Y-tube olfactometer. (A) The rate of attraction to each side (i.e. air (blue) or odor (red)) at 1, 3, 4, 6, 12 and 24 h after mosquito release in non-infected 30 mosquitoes. (B) The rate of attraction to each side at 4 h after mosquito release in non-infected 30 mosquitoes as a control. (C) The rate of attraction to each side at 4 h after mosquito release in infected 30 mosquitoes. Error bars represent the standard error of the mean. For the rate of attraction to the odor side, * indicate significant differences compared with the air side ($p < 0.05$, Tukey's honest significant difference test).

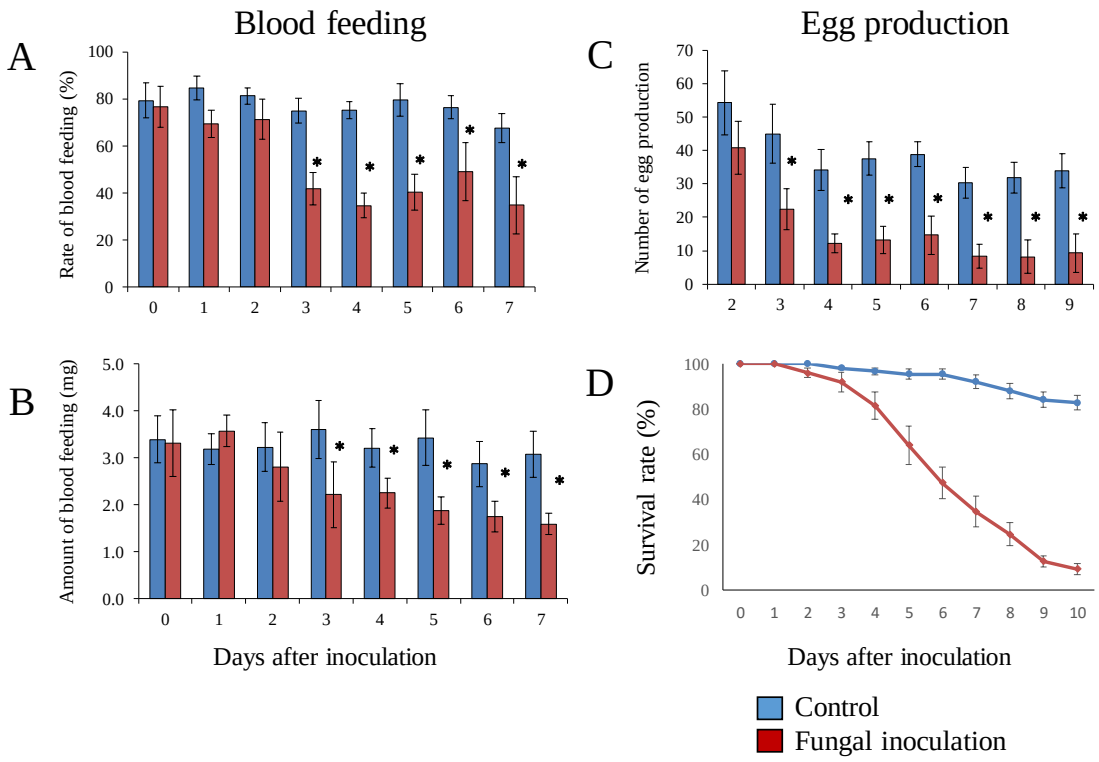

Fig. 3-6. Effect of fungal infection on blood feeding and egg production. (A) The rate of blood feeding in non-infected mosquitoes and infected mosquitoes. (B) The amount of blood feeding in non-infected mosquitoes and infected mosquitoes. (C) The number of total egg production of alive mosquitoes. (D) The average of egg production per alive mosquito. Blue and red indicate control and fungal treatment, respectively, and error bars represent the standard error of the mean.* indicates significant differences among control and fungal treatment ($p < 0.05$, Tukey's honest significant difference test).

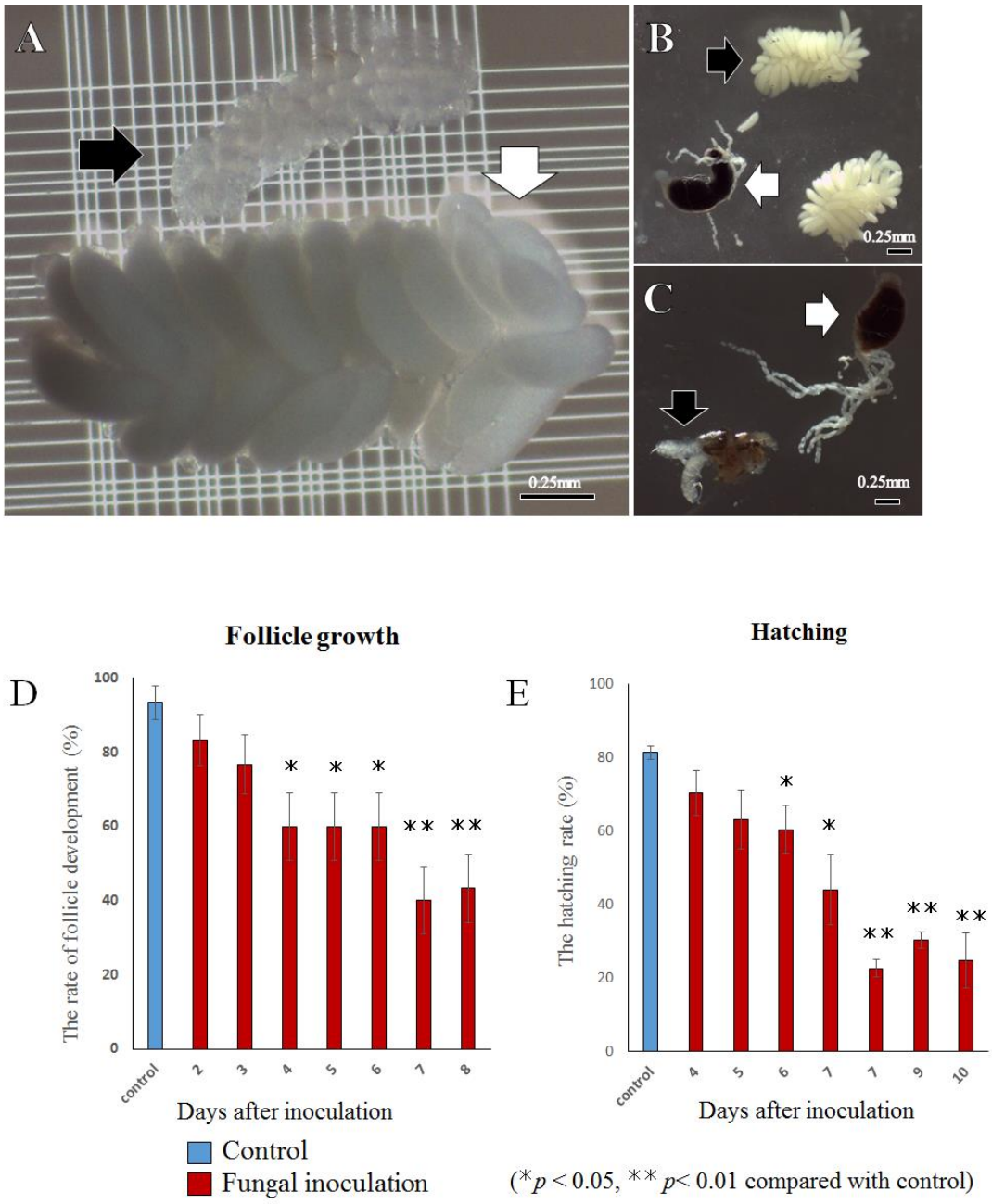

Fig. 3-7. Effect of fungal infection on follicle development and egg hatching. (A)The image of follicle development, and black and white arrow indicate non-grow follicle and grew follicle after 48 h from a blood meal, respectively. (B, C) Black and white arrow indicate follicle and midgut which took a blood meal, respectively. (B)The image of follicle in non-infected mosquito. (C) The image of follicle in infected mosquito. (D) The rate of follicle development after 48 h from a blood meal. (E) The hatching rate after 4 days from a blood meal. Blue and red indicate control and fungal treatment, respectively, and error bars represent the standard error of the mean. * indicates significant differences among control and fungal inoculation ($p < 0.05$, Tukey's honest significant difference test).

III-5. Summary

We focused on sub-lethal effect of entomopathogenic fungus *B. bassiana sensu lato* 60-2 against *An. stephensi*. The mosquitoes were inoculated with *B. bassiana s. l.* 60-2 by tarsus topical inoculation, and then host searching behaviors to heat (40°C), color (black) and odor (CO_2), the frequency and amount of blood feeding, egg production, follicle development and egg hatching were evaluated. As a result, the host searching behaviors to the heat, color and odor were significantly decreased from 3, 9, 2 days after inoculation, respectively. Also the frequency and amount of blood feeding were significantly decreased from 3 days after inoculation, and then the reduction of the number of egg production of blood fed individual was observed from 3 days after inoculation. Moreover, the follicle development and the egg hatching were significantly reduced from 5 and 6 days after inoculation, respectively. The present study suggested that *B. bassiana s. l.* 60-2 could inhibit malaria transmission and propagation of *An. stephensi* on all stages (host searching, blood feeding and egg production).

GENERAL DISCUSSION

and

FURTHER PROSPECT

In this doctoral dissertation, I focused on the relationship between the vector mosquitoes and entomopathogenic fungi. There are three main point in this study: 1) the interaction between the mosquitoes and fungi under natural condition, 2) the infection dynamism of *B. bassiana s.l.* 60-2 and lethal factor of the mosquito, and 3) sub-lethal effects of the fungus (behavioral alteration and ovipositional ability) against the mosquito. I would like discus about these phenomena and show the further prospects.

Relationship between vector mosquitoes and entomopathogenic fungi in the natural condition

There are little reports that entomopathogenic fungi were isolated from wild mosquito (Scholte et al., 2004), and the United States Department of Agriculture–Agricultural Research Service Collection of Entomopathogenic Fungi, the American Type Culture Collection, and Centraalbureau voor Schimmelcultures Fungal Biodiversity Centre listed only a few isolates of entomopathogenic fungi from mosquito family Culicidae. However, this study revealed that various spices of entomopathogenic fungi adhere and/or infect to wild mosquitoes in the natural condition (Chapter I). The latent infection rate of active mosquitoes (captured live with hand nets) collected in Japan was 4.7 %, and the fungi were isolated from puddle water at one of the mosquito breeding sites in Japan. Probably,

wild mosquitoes and entomopathogenic fungi might share a long history of association in the common habitats where they endure similar environmental conditions. In its long history, it is possible that entomopathogenic fungi develop the co-evolutionary relationship with the mosquitoes. A co-evolutionary arms race often occurs between insects and their pathogens. For instance, it was reported avoidance from entomopathogenic fungi on species within the Coleoptera, Isoptera, Hemiptera and Orthoptera (Chouvenc et al., 2008, Rath, 2000, Staples and Milner, 2000, Thompson and Brandenburg, 2005 and Villani et al., 2002). Although the exact mechanisms involved in this behavior remain unclear, Barerstock et al, (2010) conclude that insects and entomopathogenic fungi are under opposing selection pressures, and insects gain a selective advantage from detecting and avoiding fungal pathogens while successful infection of an insect by entomopathogens requires contact to be made between the host and the pathogen. On the other hand, the spores of *B. bassiana* are highly attractive to females *An. stephensi*, especially the females are preferentially attracted to dead and dying caterpillars infected with *B. bassiana*, landing on them and subsequently becoming infected with the fungus (George et al., 2013). In previous studies, Scholte et al., (2004) review that natural fungal infection on adult Anopheline mosquitoes appears relatively rare. From such a report, George et al., (2013) concluded that entomopathogenic fungi

and mosquitoes might develop only a weak co-evolutionary relationship (Scholte et al., 2004 and George et al., 2013). However, this study showed that various species of entomopathogenic fungi were isolated from wild mosquitoes. Probably, they interact each other in the natural condition, and entomopathogenic fungi influence mosquito populations in their life cycle (e.g. behavioral alteration and changing the virulence and/or specificity to adapt the mosquito host). We need more researches about the ecological and evolutional relationship between entomopathogenic fungi and the mosquitoes.

Safety of entomopathogenic fungi (especially *Beauveria* spp.) as mycoinsecticides

B. bassiana s.l. 60-2 showed high virulence and infectivity against *An. stephensi* (Chapter I), and also sub-lethal effects such as the reduction of host searching behaviors, blood feeding and egg production (Carputer III). *B. bassiana s.l.* 60-2 have strong potential for control of vector mosquitoes as microbial control agent. Therefore, in order to practical application, we should consider about safety of entomopathogenic fungi as biocontrol agent if we will use them to the mosquito control. Review on safety of entomopathogenic fungi (especially *B. bassiana*) were published by Goettel and Jaronski (1997), Goettel et al., 2001, Vestergaard et al., 2003 and Copping, 2004. In the present section, I would like consider the safety, possible side-effects of *B. bassiana* and the intended use of these

fungi as mycoinsecticides.

The primary concern is for direct effect on non-target including the following safety issues: 1) effects on non-target organisms (non-target microorganisms, plants, soil organisms, aquatic organisms, predators, parasitoids, honey bees, earth worms and nontarget arthropods), 2) effects on vertebrates (fish, amphibia, reptiles and birds), and 3) effects on mammals. It is possible that entomopathogenic fungi will be a negative influence on non-target organisms. However, Zimmermann (2007) reviewed that no serious detrimental effects have been observed after application of *Beauveria* spp. Nevertheless, to avoid possible risks, certain vertebrate pathogenicity/toxicity tests and relevant studies on non-target organisms should be made.

Additionally, although not all isolates of entomopathogenic fungi produce secondary metabolism, *Beauveria* spp. commonly produce metabolites such as beauvericin and bassianolide (Strasser et al., 2000 and Vey et al., 2001). Beauvericin is a common metabolite of many phytopathogenic *Fusarium* species and occurs in diverse foods and feeds contaminated with *Fusarium* species (Munkvold et al. 1998, Fotso et al. 2002, Logrieco et al. 2002 and Moretti et al. 2002). Research of beauvericin have demonstrated that this metabolite has insectidal, antibiotic, cytotoxic, and ionophoric properties, and also beauvericin is a specific cholesterol acyltransferase inhibitor and is

toxic towards *Artemia salina* larvae and against insect, murine and human cell lines. It can induce programmed cell death similar to apoptosis and causes cytolysis (Logrieco et al. 1998, Vey et al., 2001 and Pascale et al., 2002). In brief, in the cause of application to agriculture, we have an opportunity to take the metabolite at the least. In any case, it likely that beauvericin found and isolated from foods and feeds in nature derives from *Fusarium* spp. rather than from *B. bassiana* (Zimmermann et al., 2007). Moreover, entomopathogenic fungi could not normally infect to human. In the control of vector mosquitoes, the possibility that we influence the metabolisms of *B. bassiana* is extremely low. Based on the present knowledge it is concluded that *Beauveria* spp. are considered to be safe.

What is the trigger of behavioral alteration by entomopathogenic fungi?

Behavioral alteration (e.g., host searching and blood feeding behavior) was observed in (Chapter III), and also fungal invasion to various tissues and organ was observed in infected alive mosquitoes (Chapter II). In generically, once entomopathogenic fungi infect to their hosts, they multiply in the haemocoel in the early stage of infection, and then fungal hyphae invade all the tissues and organs after the death of the host (Liu et al., 2011, Schreiter et al., 1994, Chouvenc et al., 2009 and Toledo et al., 2010). In my study,

B. bassiana s.l. 60-2 invaded almost the tissues and organs before and after the host death. Although the mechanism of behavioral alteration by entomopathogenic fungi is unclear at the moment, such an infection property of the fungal isolates might influence the behavioral alterations. As described above Chapters, other many studies reported that fungal pathogens affect to their host behavior (Andersen et al, 2009, Bekker et al, 2014, Hughes et al, 2011, Jensen et al., 2001, Roditakis et al, 2008, Carruthers et al., 1992, Watson et al., 1993, Blanford et al., 1998 and Ouedraogo et al., 2004). However, in the almost cases, these mechanisms have not been elucidated because infection mechanisms of entomopathogenic fungi are quite complicated compared with other entomopathogens. A little studies showed that the production of metabolites and mechanical disruption of tissues by fungal growth might be responsible for the loss of appetite (Thomas et al., 1997 and Zacharuk, 1971). Additionally, Hughes (2011) reported that fungal pathogen, *O. unilateralis s.l.* infect to the head part (especially the muscles of mandible) in the alive host because it has the strategy to manipulate the behaviour of their host, *Camponotus leonardi*. Probably, fungal invasion to the organs and/or production of fungal metabolite might trigger disruption and/or inhibition of sensory organs and/or sensory neuron activities in this study.

In the further study, we will elucidate this mechanism in my laboratory. The

injection of culture filtrate of *B. bassiana s.l.* 60-2 to *An. stephensi* showed higher mortality than control. However, the mortality is not higher than the tarsus topical inoculation of *B. bassiana s.l.* 60-2 (date not shown). In brief, not only the infection of *B. bassiana s.l.* 60-2 but also the culture filtrate has some kind of influence against *An. stephensi*. In recent years, we could conduct genome editing relative easily by development of the molecular biological technique such as CRISPR/Cas9 (Zhang et al., 2014). It was known that entomopathogenic fungi produce a wide variety of compounds or metabolites (Strasser et al., 2000, Vey et al., 2001 and Roberts, 1981). If we could design "the knockout-fungal isolate of production of each compound or metabolite" and/or "gene introduction of each compound or metabolite to *Escherichia coli*", it might help with elucidation of these mechanisms.

New aspect for vector control by entomopathogenic fungi

In this doctoral dissertation, I screened the beneficial fungal isolate from the 413 isolate isolated from wild mosquitoes (Chapter I). Especially, *B. bassiana s.l.* 60-2 showed highest virulence and infectivity among them, and also has sub-lethal effects such as reduction of host searching and blood feeding behavior (Chapter III). I defined the situation that the infected mosquitoes could not transmit infectious disease, as "death as

a vector" in contrast to "death of a vector". In order to reduction of host searching and blood feeding behavior have a potential for prevent malaria transmission, we should more precisely evaluate about sub-lethal effects. However, it is difficult for evaluating only the sub-lethal effects because *B. bassiana s.l.* 60-2 have high virulence and infected mosquitoes die early. Ouedraogo et al., (2004) concluded that more detailed evaluations of sub-lethal effect (i.e. reduction of feeding activity) over a longer period of time would be necessary to assess more precisely the effect of non-lethal infection. Reyes-Villanueva et al., (2011) demonstrated that low virulence isolate induced in the fungus-inoculated male a higher aggressiveness for inseminating females to such an extent that the sexual activity in contrast this phenomenon was not occurred in high virulence isolate. Accordingly, we need the isolate which have low virulence but high infectivity, and have the sub-lethal effects against *An. stephensi*. Fortunately, I have 413 fungal isolates isolated from wild mosquitoes, and some of isolates showed low virulence but high infectivity among them (Chapter I). In the further study, we should focus the sub-lethal effects of the fungal isolate which showed low virulence. The application of the low virulence isolate might be able to clearly evaluate the sub-lethal effects and its mechanisms, and it will more develop the vector control.

Resistance to microbial control agents

It seems like we have a seriously problem about killing the insect pests. As stated in the general introduction, resistance to chemical insecticides is a major problem for the controlling insect pests. As an alternative approach, microbial control agents such as bacteria, viruses and fungi were used for various studies. However, in recent years, few studies have described the development of resistance for microbial control agents. Although *Bacillus thuringgiensis* (*Bt*) is used as biological control agent worldwide, Siegwart et al. (2015) demonstrated that at least 27 species of insects have potential for resistance to *Bt* under laboratory conditions, and these resistances mainly concern Lepidoptera (Pardo-Lopez et al., 2013). In the field study, 3 species of Lepidoptera, *Plutella xylostella*, *Trichoplusia ni*, and *Plodia interpunctella*, have shown resistance to *Bt*-formulation (Tabashnik, 1994; Janmaat and Myers, 2003; McGaughey, 1985). Furthermore, in the study of controlling mosquitos, Mittal et al., (1998) demonstrated variations in the degree of response of different strains of *An*. *stephensi* against *Bacillus sphaericus* (*Bs*) toxins and the development of a high degree of resistance to *Bs* within four generations. Paris et al. (2011) reported that persistence of *Bacillus thuringgiensis israelensis* (*Bti*) in the environment induces resistance to multiple *Bti* toxins in mosquitoes, and up to 30-fold resistance to each individual *Bti* toxin can be attained after

a few generations of selection. And also in the study of viruses, the larvae of Lepidoptera have resistance to infection of nuclear polyhedrosis virus, and the resistance variation for 20 insect species was summarized by Briese (1986). The velvet worm (*Anticarsia gemmatalis*) which collected from Brazil and the USA could develop resistance to alphabaculovirus in laboratory conditions (Abot et al., 1996). In the study of fungi, it was reported that insects have defense mechanisms to the fungi, mainly protease inhibitors, such as serine protein inhibitor, trypsin inhibitors, chymotrypsin inhibitors, elastase inhibitors and subtilisin inhibitors (Siegwart et al. 2015). Under constant selective pressure from *B. bassiana*, 25th generation larvae of Greater wax moth, *Galleria mellonella*, exhibited enhanced resistance a little, which was specific to *B. bassiana* and not to *M. anisopliae* (Dobovskiy et al., 2013). In brief, lethal effect causes development of the resistance to also microbial control agent. Almost insects are unlikely to develop the resistance to entomopathogenic fungi compared with other entomopathogens because their mode of actions is quite complicated. However, a "no risk" situation does not exist in development of the resistance, certainly not with chemical pesticides and other pathogens, and even with fungal pathogens could not absolutely prove a negative. Conventional vector control method was evaluated only lethal effect, and it has been occurred the selection pressure. On the other hand, low virulence isolates are not induced

the resistance development because of non-secretion pressure for fungus-resistance genes. If we will obtain the special isolates which show low virulence and have the sub-lethal effects, the isolates might could prevent the malaria transmission as “death as a vector” excluding possibility of the resistance development.

Conclusion

B. bassiana s.l. 60-2 showed sub-lethal effects against *An. stephensi* (e.g. reduction of host searching and blood feeding behavior) in (Chapter III). Moreover, entomopathogenic fungi showed the sub-lethal effect against *Ae. aegypti* in my study. A total of 11 fungal isolates showed higher infectivity against *Ae. aegypti* among the original fungal library (Chapter I), and 3 isolates (*B. bassiana* and *Lecanicillium* sp.) had tendency to decrease their host searching behavior to the heat (date not shown). These results suggested that entomopathogenic fungi have potential for inactivation of various disease vectors. So our future research will be focused on “evaluation of disease transmission risk by behavioral alterations” and “investigation of these mechanisms by entomopathogenic fungi infection against various disease vectors”. This finding, “inactivation of transmission capability of disease vector by entomopathogenic fungi”, could apply to various vector control.

Acknowledgements

The research works carried out at Laboratory of Environmental Microbiology, Obihiro University of Agriculture and Veterinary Medicine. This study was supported by JSPS KAKENHI Grant Number 21880004 and 25850227 to D. A., and the International Collaborative Research Program for Tackling the NTDs Challenges in African countries from Japan Agency for Medical Research and development, AMED, to H. K., and Grant-in-Aid for JSPS Fellows Grant Number 26-8772 to M. I.

I am deeply indebted to Professor Masanori Koike, my supervisor at Laboratory of Environmental Microbiology, for accepting me in this laboratory and his universal supports.

I am deeply indebted to Assistant Professor Daigo Aiuchi, co-supervisor at Laboratory of Environmental Microbiology, for his passionate guidance and intellectual creativity on my study life.

I am grateful to Professor Shu Hase, Yamagata University, for his valuable suggestions on my graduate program.

My special thanks to Professor Hirotaka Kanuka, Department of Tropical Medicine, Tokyo Jikei University School of Medicine, for accepting me on the internship in his laboratory, and technical advice and useful suggestions.

I would like to thank to Professor Masayuki Tani, Laboratory of Environmental Microbiology, Obihiro University of Agriculture and Veterinary Medicine, and Professor Ryutaro Maeda, Obihiro University of Agriculture and Veterinary Medicine, for their valuable suggestions and comments from different angle to my studies.

I am very grateful to all other members of Laboratory of Environmental Microbiology, especially Yu Matsuzaki and Chika Hosoya for their support and friendship.

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Abstract (要旨)

昆虫寄生菌は自然界に普遍的に存在する微生物であり、その長い歴史の中で宿主昆虫と密接な関係を築き上げてきた。感染症を理解する上で媒介者として重要な役割を担う節足動物（ベクター）もまた、自然界において昆虫寄生菌と密接な関係を築き上げてきたと考えられる。ハマダラカ属の蚊によって媒介されるマラリアは、年間 58 万人もの死者を出すことから世界的な脅威となっている。マラリア防除は、主に媒介者であるハマダラカを合成化学殺虫剤で殺すことにより防除されてきたが、薬剤的抵抗性の発達が世界中で問題となっていることから代替防除技術の確立が急務とされている。一方で、近年では昆虫寄生菌による感染症媒介蚊の防除研究が注目されている。しかしながら、これらの研究は蚊類を除いた昆虫由来の菌株を使用しており、昆虫寄生菌が自然界で蚊にどのような影響を与えているかは不明なままである。また、これまでに成虫蚊に感染した昆虫寄生菌の感染動態を解明した研究もない。さらに、昆虫寄生菌は様々な宿主昆虫の行動を変化させることも知られている。本研究では、自然界における蚊と菌の関係を明らかにするとともに、昆虫寄生菌が蚊にどのように感染するかを解明する。加えて、行動学的なアプローチから菌感染蚊の病原体媒介能を評価することで、感染症媒介蚊と昆虫寄生菌の関係を解明し、新たな防除法を視野に入れた研究を展開する。

はじめに、自然界における蚊と昆虫寄生菌の関係を明らかにするために、日本および西アフリカに位置するマラリア流行国のブルキナファソにおいて野生蚊の採集を行った。採集された 3000 頭以上の野生蚊から昆虫寄生菌の分離を試みた結果、計 413 菌株の昆虫寄生菌が分離された。これらの菌をハマダラカに対して接種すると、病原性や感染性の高い菌株の検出に成功した。特に *Beauveria bassiana sensu lato* 60-2 は半数致死日数 5.8 日を示し、他の研究報告と比較しても非常に病原性の高い菌株であった。これらの結果から、自然界において昆虫寄生菌は普遍的に蚊に付着もしくは感染しており、それらの中には蚊類の防除に特化した菌株が存在することが明らかとなった。

次に、昆虫寄生菌の感染動態の解明のため、*B. bassiana s.l.* 60-2 をハマダラカに跗節局所的に接種し、菌感染蚊のパラフィン切片をグロコット染色することで菌の感染動態を観察した。その結果、菌の侵入は跗節だけではなく口吻でも起きており、その後菌が他の部位に比べて頭部へ早く侵入する傾向が観察された。また、一般的に昆虫寄生菌はまず宿主の血体腔内で増殖し、宿主の死後に様々な組織・器官へ侵入するとされてきていたが、本研究では宿主が生きている感染初期の段階から *B. bassiana s.l.* 60-2 が様々な器官・組織へ侵入していることが明らかとなった。さらに、菌の脳への感染と死亡率に高い相関がみられた上、死個体の頭部を観察すると全ての個体に菌の脳感染が起きていた。最後

に、ハマダラカの跗節もしくは口吻それぞれに対して菌を接種し、ハマダラカの生存率を比較すると、口吻経由の接種が跗節経由に比べハマダラカを早期に死に至らしめた。以上の結果から、跗節局所的接種方法は跗節および口吻で菌の感染が起き、特に口吻経由による頭部（さらに脳）への感染が、蚊類の防除にとって重要であることが示唆された。

以上で得られた知見（*B. bassiana s.l.* 60-2 の頭部への感染が早いことや、宿主が死ぬ前に菌が様々な器官・組織に侵入すること）や、昆虫寄生菌が様々な宿主昆虫の行動に変化をもたらす研究報告を基に、本研究では *B. bassiana s.l.* 60-2 に感染したハマダラカのマラリア伝播能に関わる行動（宿主探索行動・吸血行動）や繁殖能（産卵・濾胞発育）に着目した。熱および色に対する宿主探索行動の評価には自動行動アッセイ装置を用い、においに対する宿主探索行動には Y 字管型オルファクトメータを用いて評価した。その結果、菌感染蚊は健全個体に比べて全誘引源に対する誘引性が減少し、特に熱およびにおいに対しては感染初期（2，3 日目）から劇的に誘引性が減少する結果となった。また、脱繊維血に対するハマダラカの吸血およびその後の産卵数を評価した結果、菌感染蚊は吸血率・吸血量・産卵数がともに健全個体と比べて有意に減少することが明らかとなった。さらに、吸血済みの菌感染蚊における濾胞発育を評価すると、濾胞の発育が全く見られない個体が観察された。健全個体と比べて、菌感

染蚊は菌感染 4 日目から濾胞発育率が有意に減少し、その後産卵された卵の孵化率も減少する結果となった。昆虫寄生菌がハマダラカに感染することは、宿主探索行動や吸血行動の阻害によりマラリア媒介能が劇的に低下するだけでなく、繁殖能が低下することでポピュレーションを徐々に縮小できることから間接的にマラリア媒介能を抑える可能性が示唆された。

これまで昆虫寄生菌の防除効果には致死効果つまり「ベクターを殺す」ことに焦点が置かれてきた。本研究により、昆虫寄生菌と感染症媒介蚊の関係を解明した結果、昆虫寄生菌によりベクターの媒介能が不活性化されるという知見が得られた。これらの知見を蚊の防除研究に導入することで、革新的な防除技術の確立につながると考えられる。