ผลของสารอาหารต่อลักษณะเฉพาะเชิงสัณฐานและการแข่งขันระหว่างเพศผู้ของด้วงคีมร่องเก่า *Aegus chelifer chelifer* MacLeay, 1819

นายณัฏฐ์ ทรงวรวิทย์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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NUTRITIONAL EFFECTS ON MORPHOLOGICAL CHARACTERISTICS AND MALE-MALE COMPETITION OF STAG BEETLE *Aegus chelifer chelifer* MacLeay, 1819

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A Dissertation Submitted in Partial Fulfillment of the Requirements
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ณัฏฐ์ ทรงวรวิทย์ : ผลของสารอาหารต่อลักษณะเฉพาะเชิงสัณฐานและการแข่งขันระหว่างเพศผู้ของด้วงคีมร่องเก่า *Aegus chelifer chelifer* MacLeay, 1819 (NUTRITIONAL EFFECTS ON MORPHOLOGICAL CHARACTERISTICS AND MALE-MALE COMPETITION OF STAG BEETLE *Aegus chelifer chelifer* MacLeay, 1819) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. ชัชวาล ใจซื่อกุล, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ดร. บัณฑิกา อารีย์กุล บุทเชอร์, 254 หน้า.

ด้วงคีมมีความแปรผันทางด้านขนาดลำตัวและอาวุธสูง โดยปัจจัยหลักที่ส่งผลคือสิ่งแวดล้อมในระยะตัวอ่อน แต่สิ่งแวดล้อมมีอิทธิผลต่อความแปรผันเหล่านี้ได้อย่างไรนั้นยังไม่เป็นที่แน่ชัด และยังรวมไปถึงความเชื่อมโยงระหว่างสิ่งแวดล้อม ความแปรผันเชิงสัณฐานภายนอกและพฤติกรรม ที่ยังมีการศึกษากันไม่มากนักโดยเฉพาะในด้วงคีม งานวิจัยนี้เริ่มจากการสำรวจด้วงคีมในธรรมชาติภายในป่าดิบแล้งแห่งหนึ่งในจังหวัดจันทบุรี พบว่าการปรากฏของด้วงคีมในขอนไม้ และจำนวนที่พบมีมากในขอนไม้ที่มีระดับความผุปานกลาง และมีปริมาณไนโตรเจนและมวลชีวภาพของเห็ดราที่สูง การทดลองในห้องปฏิบัติการได้เลือกด้วงคีมร่องเก่า *Aegus chelifer chelife*r MacLeay, 1819 เป็นตัวแทนในการศึกษา ความสัมพันธ์เชิงแอลโลเมตรีระหว่างความยาวปีกคู่หน้าและคีมในด้วงเพศผู้ มีลักษณะใกล้เคียงกับ piecewise linear model มากที่สุด และยังแสดงภาวะทวิสัณฐานในด้วงเพศผู้อีกด้วย ไม่พบ narrow-sense heritability ของขนาดตัวและความยาวคีมของด้วงที่ได้จากการเพาะเลี้ยง แต่การเปรียบเทียบขนาดตัวและแอลโลเมตรีระหว่างประชากรด้วงจากกรุงเทพมหานครและปริมณฑล และประชากรด้วงจากจังหวัดจันทบุรีพบว่ามีความแตกต่างอย่างมีนัยสำคัญ ปริมาณอาหาร ปริมาณไนโตรเจน และการหมักของผงขี้เลื่อยที่ใช้เลี้ยงด้วงมีผลต่อการเจริญของตัวอ่อนและขนาดตัวเต็มวัย ในขณะที่สารที่เพิ่มในอาหารไม่มีผลอย่างมีนัยสำคัญ ยิ่งกว่านั้นการเลี้ยงตัวอ่อนด้วยผงขี้เลื่อยที่มีเห็ดราย่อยไม้ให้ผลเชิงลบต่อการเจริญของด้วง ด้วงตัวเต็มวัยเพศผู้ขนาดใหญ่มีโอกาสชนะการต่อสู้มากกว่าด้วงขนาดเล็ก โดยความกว้างของอกปล้องแรกเหมาะเป็นตัวทำนายผลการต่อสู้มากที่สุด โดยสรุปปริมาณของอาหารที่ตัวอ่อนด้วงได้รับมีส่วนสำคัญต่อขนาดของตัวเต็มวัยและผลการต่อสู้ของด้วงคีมร่องเก่ามากกว่าคุณภาพของอาหาร ซึ่งผลเช่นนี้อาจรวมไปถึงด้วงคีมชนิดอื่น ๆ หรือแมลงกินไม้ที่มีพฤติกรรมการแข่งขันกันระหว่างเพศผู้ด้วยกัน

ภาควิชา ชีววิทยา	ลายมือชื่อนิสิต .....
สาขาวิชา สัตววิทยา	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
ปีการศึกษา 2560	ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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KEYWORDS: DECAYING WOOD / ALLOMETRY / BODY SIZE / GROWTH / MANDIBLE / FIGHT

NUT SONGVORAWIT: NUTRITIONAL EFFECTS ON MORPHOLOGICAL CHARACTERISTICS AND MALE-MALE COMPETITION OF STAG BEETLE *Aegus chelifer chelifer* MacLeay, 1819. ADVISOR: ASST. PROF. CHATCHAWAN CHAISUEKUL, Ph.D., CO-ADVISOR: ASSOC. PROF. BUNTIKA AREEKUL BUTCHER, Ph.D., 254 pp.

Stag beetles have great intraspecific variation in their bodies and weapon sizes, which are strongly influenced by the environment during the larval stage. However, the impacts of environmental factors affect on such variation and the links between environmental factors, morphological characteristics and behaviours are still unclear. This study first surveyed stag beetles discovered in natural habitat of a dry-evergreen forest in Chanthaburi province, Thailand, to roughly examine possible factors relating to growth of stag beetles. The occurrence and numbers of stag beetle larvae found in logs were high in those of a moderate decay class with relatively high nitrogen content and fungal biomass. For laboratory experiments, *Aegus chelifer chelifer* MacLeay, 1819 was used as the representative species for this study. Allometric relationship between elytra and mandible length of male beetles best fitted to the piecewise linear model and revealed the existence of dimorphism in males of this species. Narrow-sense heritabilities of mandible length and adult body size of stag beetles from captive breeding were not detected. However, comparisons of beetles between Bangkok metropolitan population and Chanthaburi population showed significant differences in body size and allometry. Testing for the effects of diet indicated that diet quantity, nitrogen content and fermentation of sawdust-based diet had significant effects on larval performances and adult body size. Furthermore, rearing of larvae with sawdust infested by wood-decaying fungi showed negative effects on the larval growth. For behavioural study, larger males showed a higher chance to be winners from male-male combat and pronotum width was the best predictor for the fighting outcome. Quantity of diets had more important effects on body size and intraspecific outcomes than diet quality in *A. chelifer chelifer*, and possibly in other stag beetles or other saproxylic insects with high male-male competition.

Department:	Biology	Student's Signature	.....
Field of Study:	Zoology	Advisor's Signature	.....
Academic Year:	2017	Co-Advisor's Signature	.....

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CHAPTER I

INTRODUCTION

Since Charles Darwin proposed the theory of evolution by natural selection, it has become the main and fundamental dogma in the field of biology (Darwin, 1859). Many biologists have then attempted to comprehend and expand this theory to various kinds of animals to explain the mechanisms behind this theory. Sexual selection, a mode of natural selection, has proved to explain sexual dimorphism in many animals, including stag beetles, with shaping their morphological characteristics to have extraordinary structures, particularly exaggerated mandibles in males (Kawano, 2000; Hosoya and Araya, 2005; Harvey and Gange, 2006; Bonduriansky, 2007; Emlen et al., 2012).

Many beetles, such as rhinoceros, dung and stag beetles, exhibit intraspecific variations in body size and their secondary sexual traits (Shiokawa and Iwahashi, 2000a; Kawano, 2002; Moczek, 2002; Kawano, 2003; Harvey et al., 2011a; Iguchi, 2013). Studies on these insects revealed that morphological variations of the adults were mainly the result of physiological responses affected by external environment during larval stage (Moczek, 1998; Shafiei et al., 2001; Moczek, 2002; Karino et al., 2004; Okada and Miyatake, 2010; Gotoh et al., 2011; Hardersen et al., 2011; Gotoh et al., 2014; Romiti et al., 2017). These variations are important for male beetles regarding sexual selection. In rhinoceros beetles, body size and horn length were reported as crucial components to determine the outcome of fight between males, including their

fighting and mating behaviours (Siva-Jothy, 1987; Karino et al., 2005; Okada and Hasegawa, 2005; Harvey and Gange, 2006; Inoue and Hasegawa, 2013).

Male stag beetles of many species have great variations in body size and mandible length. The largest males can have the body size nearly up to three times of the smallest males (Harvey and Gange, 2006; Harvey et al., 2011a). However, information about the effects of nutrition on morphological characteristics and fighting behaviour of male stag beetles are very limited. Therefore, the objectives of this dissertation are as follows:

1. To investigate the effects of nutritional factors on morphological variation of adult stag beetles.
2. To investigate the importance of morphological variation to male-male competition of stag beetles.

This study began by observing stag beetles in natural habitats. Presence of stag beetles in decaying logs in a dry-evergreen forest was examined to know the properties of wood that female stag beetles used as criteria for oviposition selection (Chapter III). The obtained results were then used as a guideline for subsequent studies about the diet properties that might associate with the growth and size variation of stag beetles. Then, *Aegus chelifer chelifer* MacLeay, 1819, a small tropical stag beetle, was selected as a representative stag beetle to study. This species is classified in:

Phylum: Arthropoda

Class: Insecta

Order: Coleoptera

Family: Lucanidae

Genus: *Aegus*

Species: *Aegus chelifer* MacLeay, 1819

Subspecies: *Aegus chelifer chelifer* MacLeay, 1819

A. chelifer chelifer stag beetles are suitable as a study model because of many reasons, namely (1) its sexual dimorphism and great size variation in males, (2) ability to be bred under artificial conditions and relatively short life cycle, approximately 3−4 months from eggs to adults, (3) its status as the most common stag beetle species found in Thailand with a large number of specimens in natural habitats, and (4) its wide distribution throughout the mainland of Southeast Asia, both in forest and urban areas, and they are reported as an alien species in other regions, indicating high adaptation to live under various environmental conditions (Mizunuma and Nagai, 1994; Pinratana and Maes, 2003; Ek-Amnuay, 2008, 2009; Carpaneto et al., 2010). Unfortunately, there was no report about the use of *A. chelifer chelifer* in any research before and thus lack of basic information both genetic and environmental effects on phenotypic variation.

External morphological characteristic, i.e. body size, mandible size and allometry, of *A. chelifer chelifer* were examined to estimate the degree of phenotypic variation of stag beetles both within and between populations using wild specimens and stag beetles from captive breeding (Chapters IV and V). Then, the possibility of food properties that might influence body size variation in adults was examined by rearing them with manipulated diets under laboratory conditions (Chapters VI and VII). Lastly, the importance of adult size to their behaviours was examined by focusing on male-male interactions to understand the role of adult size variation on sexual selection of stag beetles (Chapter VIII). The study of morphological variation in stag beetles could

help to improve understanding in the mechanism of body size variation and demonstrate the relationships between nutrition, morphology and sexual selection.

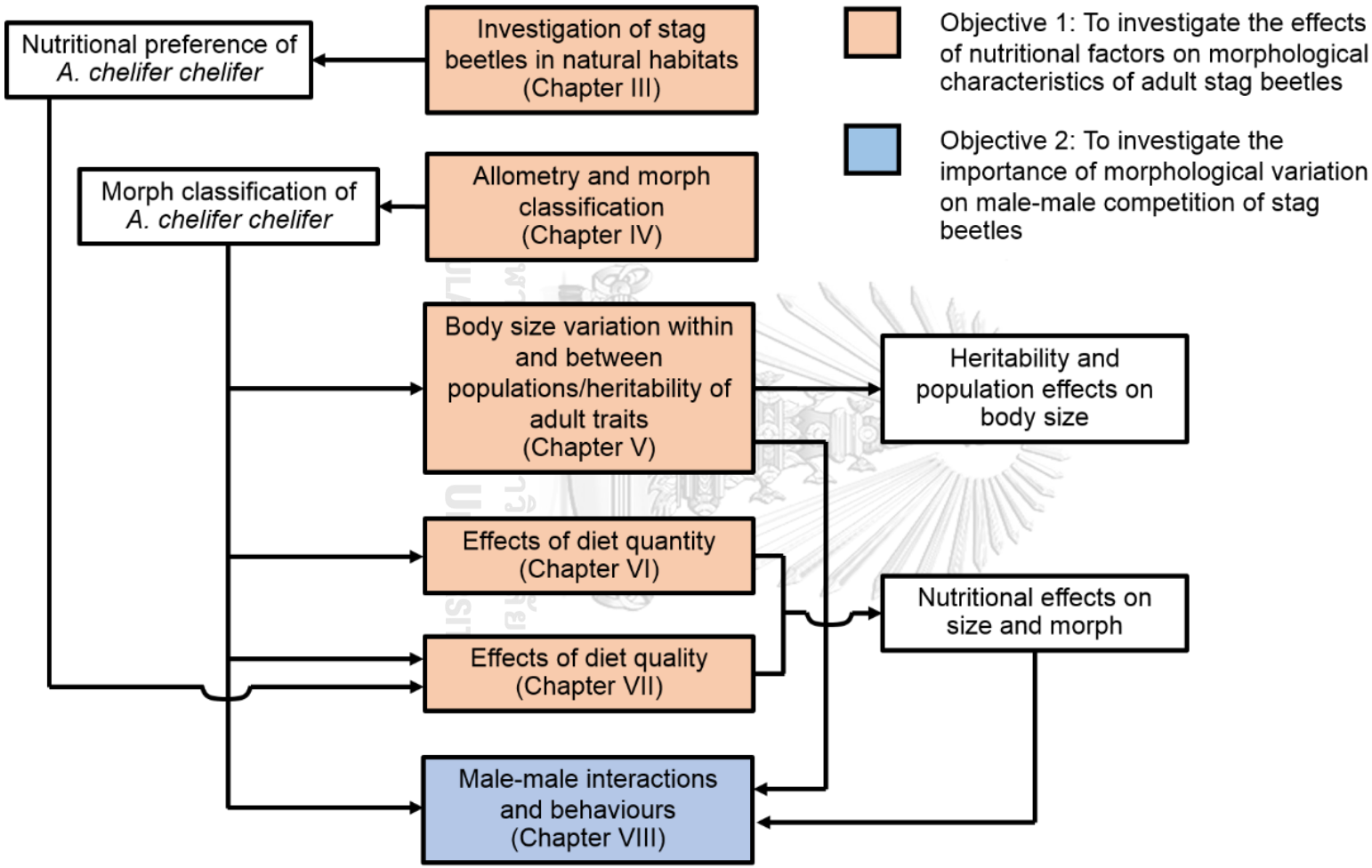

Figure I-1 Research scheme.

CHAPTER II

LITERATURE REVIEWS

Biology of Stag Beetles

Stag beetles belong to the order Coleoptera, family Lucanidae. Most males have long projecting mandibles which are used as weapons for male-male competition. In contrast, female stag beetles have relatively smaller body sizes and have relatively shorter mandibles. According to the taxonomic classification of Crowson (1956), stag beetles can be identified from their antennae which are geniculate with 3–5 expanded apical flagellomeres to form a lamellate club (Figure II-1A), and their abdominal sternites consist of five visible segments (Figure II-1B). Stag beetle larvae have soft whitish C-shape body (scarabaeiform or grub) with longitudinal or Y-shaped anal opening (Figure II-1C). Head capsule is rigid with yellow or orange. There are stridulatory organs presented on mesothoracic and metathoracic legs (Figure II-1D) (Ritcher, 1967). Sex of stag beetle larvae can be identified from the presence of yellow ovoid shape of ovaries in females which are visible through larval cuticle at the dorsal part of abdomen (Fremlin and Hendriks, 2014) (Figure II-2). There are approximately 1,200 described species of stag beetles around the world (Mizunuma and Nagai, 1994). Stag beetles are distributed in the tropical, sub-tropical and temperate regions. Over 60% of all stag beetle species can be found in the Southeast Asia (Kameoka and Kiyono, 2003). Of these, one hundred and two species from 24 genera have been recorded from Thailand (Pinratana and Maes, 2003). Adults feed on sap exudate on tree trunks and

rotten fruits while larvae act as saproxylic insects feeding on decaying wood of broad-leaf trees (Araya, 1993b; Harvey et al., 2011a).

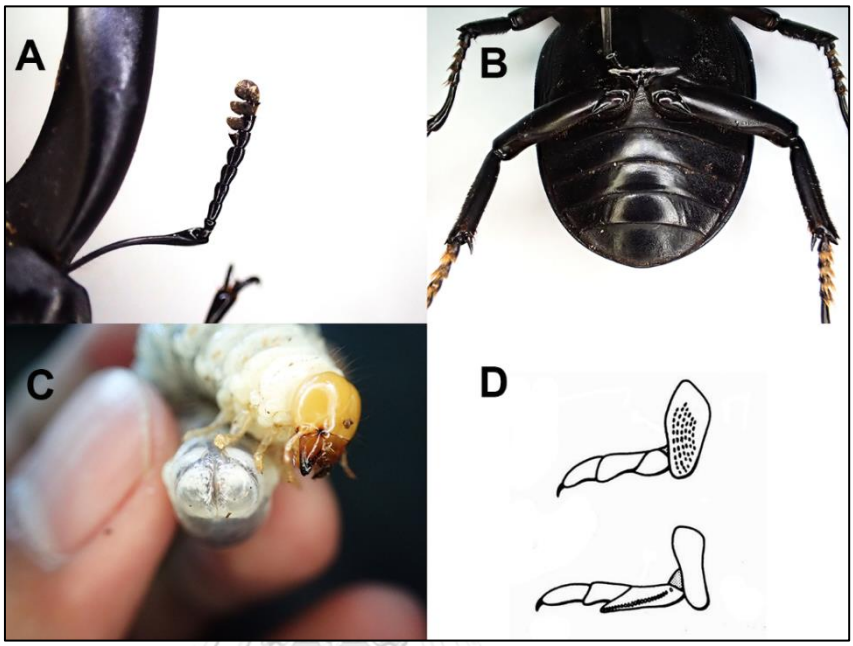

Figure II-1 Important morphological characteristics to identify adult and larval stag beetles, (A) antenna as geniculate with 3–5 expanded apical flagellomeres to form a lamellate club, (B) five visible abdominal sternites, (C) vertical anal opening, (D) stridulatory organs on mesothoracic and metathoracic legs of stag beetle larva (picture from Ritcher, 1967).

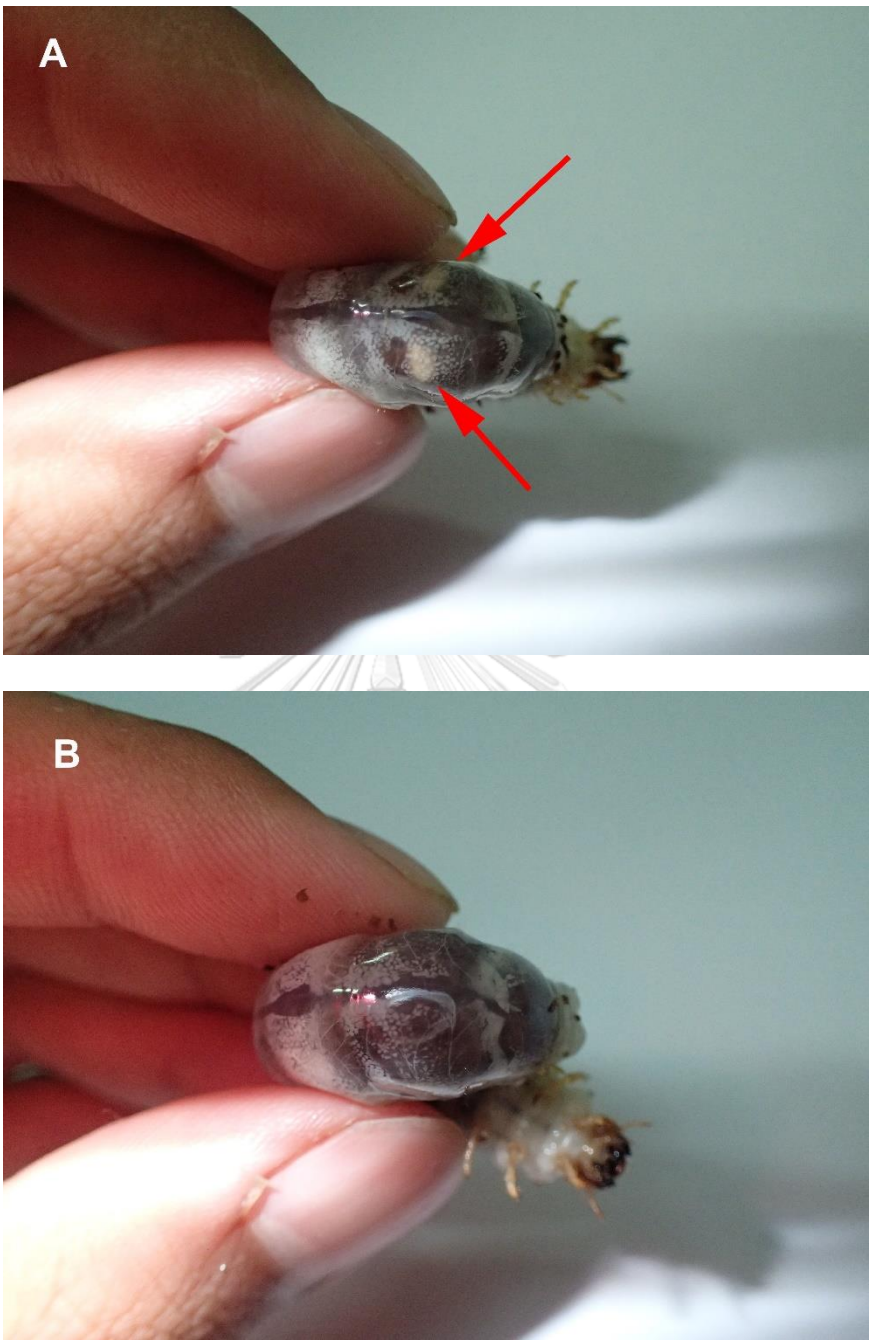

Figure II-2 Sexual difference between female (A) and male (B) stag beetle larvae. Female larvae have a pair of yellow ovoid shape of ovaries visible through larval cuticle at the dorsal part of abdomen (arrows), while males lack this characteristic.

Intraspecific Variations in Size and Shape

Intraspecific variation is a common phenomenon found in various kinds of organisms, which is influenced by both genotypic variation and environmental induction (Scheiner, 1993; Emlen and Allen, 2003; Lewis et al., 2012; Tsuchiya et al., 2012). Genotypic variation has an important role in generating varieties of morphological characteristics through natural selection. Morphological characteristics of special structures, especially allometry, in insects may be influenced by genetic factors. Studies on dung beetle and stalk-eyed fly showed that their allometry between length of exaggerated structures and body size could be changed by artificial selection (Wilkinson, 1993; Emlen, 1996). For stag beetles, the research by Gotoh et al. (2012) in *Cyclommatus metallifer* Boisduval, 1835 showed that absolute mandible length was not heritable, but there was significant heritability of static allometry between mandible length and body size.

In holometabolous insects, there is evidence that environmental factors, especially nutrition during larval period, are the major components determined the morphology of adults by regulating at time period before moulting and during metamorphosis (Nijhout, 1975; Shafiei et al., 2001; Cook and Bean, 2006). Positive correlation between nutritional qualities and body sizes or special structures was reported in rhinoceros beetles, *T. dichotomus*, dung beetles, *Onthophagus taurus* (Schreber, 1759) and stag beetles, *C. metallifer* (Iguchi, 1998; Moczek, 1998; Shafiei et al., 2001; Karino et al., 2004; Gotoh et al., 2011).

In *O. taurus*, two distinctive characteristics of horned (major morph) and hornless (minor morph) males can be found within the same population. Only male

larvae that gain grown weight higher than a critical point before pupation would become horned males. In contrast, larvae in habitats with low food availability habitat could not reach the critical weight and became hornless males (Moczek, 1998). Because environment is the main factor influencing morphological variations in these beetles, this indicates that the variation is the result of phenotypic plasticity. Several experiments to reveal the mechanism behind phenotypic plasticity were also conducted. The results showed that nutritional supply associated with insect hormones, especially juvenile hormones which acts on adult structural development during the last instar (Emlen and Nijhout, 1999, 2001; Shelby et al., 2007; Whitman and Ananthakrishnan, 2009).

Decaying Wood and Saproxylic Insects

The decomposition of dead wood is an essential process in ecosystems (including nutrient recycling) and in turn is an important natural resource in terrestrial ecosystems where it acts as a microhabitat and reservoir for many living communities, especially invertebrates and microorganisms (Yee et al., 2001; Kehler et al., 2004; Nordén et al., 2004; Lachat et al., 2012). Saproxylic insects (insects feed on dead wood or wood remains) are the dominant invertebrates that utilize plant remains efficiently.

Feeding on decaying wood, stag beetle larvae must face with a major problem of nutritional availability, same as many other wood-feeding (or xylophagous) insects. Wood generally consists of three main components; cellulose, hemicelluloses and lignin (Schmidt and Czeschlik, 2006). Although wood contains high carbohydrate, its constituents are indigestible. Moreover, nitrogen is usually present in a very low level and insufficient for insect growth (Ayres et al., 2000; Schmidt and Czeschlik, 2006;

Tanahashi et al., 2009). Therefore, xylophagous insects have diverse strategies to overcome these problems. One strategy that can be found in various insects is the association with fungi. Bark beetles and some eusocial insects, such as termites and leafcutter ants, are good examples of mutualistic relationships with the fungi (Ayres et al., 2000; Hyodo et al., 2000; Solomon et al., 2004). It is believed that fungi enhance digestibility of plant components for insects by altering lignocellulosic components to be easily digestible forms or suitable forms for assimilation (Hanula, 1996). Stag beetle larvae were also associated with decaying wood affected by wood-decaying fungi (Araya, 1993b). The growth rate of the stag beetle larvae was positively correlated with the nitrogen content or C/N ratio in fungal mycelium, indicating that nitrogen and fungi were important elements in determining of growth (Tanahashi et al., 2009).

Male-Male Competition

Competition in order to claim limited resources, especially for mating, is commonly found in many animals. Competition between males or male-male competition is a mode of sexual selection that males compete one another for access females. By sexual selection, males of many animals evolve to possess some traits for increasing their mating success, such as horns of rhinoceros beetles and mandibles of stag beetles (Emlen and Nijhout, 2000; Pomfret and Knell, 2006; Bonduriansky, 2007).

Agonistic interactions of animals can be exhibited in various forms, such as displaying a certain performance to tell their strength and quality, engaging in direct conflict, or performing a series of behaviours from the lowest to highest aggressive intensity, that depend on species, resource value, resource holding potential (RHP), experience and mechanism to obtain information during the interactions (Siva-Jothy,

1987; Payne and Pagel, 1997; Hofmann and Schildberger, 2001; Pratt et al., 2003; Jennings et al., 2004; Goyens et al., 2015b). Under symmetric resource value, individuals with larger body size normally win the combat, but other traits, especially secondary sexual traits, can also influence to the outcome and may be used as a predictor if those traits are reliable to indicate fighting potential, for examples eyespan of stalk-eyed flies, horn length of Japanese horned beetles and chelae of shore crabs (Sneddon et al., 1997; Karino et al., 2005; Small et al., 2009).

Normally, fight between two individuals is terminated by the individual which gives up from the fight sooner, or known as “loser”. From the behavioural game theory perspective (Smith, 1982), relations of fight intensity and fight duration to RHP of the contestants are of interest topics about how decisions to escalate and give up from the fight are made (Briffa, 2014). According to Arnott and Elwood (2009), assessment strategies of fighting ability could be classified into three main mechanisms, based on the gathering information methods by contestants as follows. (1) Pure self-assessment, which each contestant knows only its own abilities and the decision to give up is made when the cost of fight of one contestant (loser) exceeds a threshold. (2) Cumulative assessment, which is similar to the pure self-assessment, but the actions of the opponents can inflict on the contestants resulting in acceleration of the cost reaching to the threshold point. Individuals which have higher threshold and/or better ability to inflict the cost of the opponents will be winners. (3) Mutual assessment, which each contestant assesses opponent’s RHP relative to their own. Thus it gives advantages to both contestants by reducing the cost of fight when an asymmetric combat takes place.

Economic Importance of Stag Beetles

Stag beetles are popular among insect enthusiasts. Many people prefer to rear them as pet. In Japan, the market size of stag beetles was about 100 million US dollars, and more than 1 million beetles were imported annually mostly from Southeast Asian countries, especially Indonesia, the Philippines and Thailand, both legally and illegally (Goka et al., 2004; Tournant et al., 2012). Price of stag beetles could vary from a few US dollars to 100,000 US dollars (New, 2005). The size of beetles was an important factor to determine price, which is increased if that insect is larger. For example, from the survey of Kameoka and Kiyono (2003), *Dorcus antaeus* Hope, 1842, had a price range from 37.6 to 3,344.5 US dollars and *D. curvidens* (Hope, 1840) had a price range from 8.4 to 1,087 US dollars. Even though captive breeding of stag beetles has already been done successful, the production of these beetles with larger size to satisfy the massive market demand is still a challenge.

CHAPTER III

DECAYING WOOD PREFERENCE OF STAG BEETLES (COLEOPTERA: LUCANIDAE) IN A TROPICAL DRY-EVERGREEN FOREST

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Abstract

Larvae of many insect species, including stag beetles, have a limited mobility from their initial oviposition site. The fate of immature stages, therefore, depends on the maternal choice of oviposition site. Decaying wood preference by stag beetles was studied in a dry-evergreen forest in Chanthaburi province, Thailand. From a total of 270 examined logs, 52 contained stag beetles (255 total), which were identified to eight species from five genera. *Aegus chelifer chelifer* MacLeay, 1819 was the dominant species both by occurrence and by number of individuals. The occurrence and numbers of stag beetle larvae found in logs was more frequent in those of a moderate decay class (Class II−IV), which had moderate hardness and water content. Principal component analysis (PCA) revealed that logs with stag beetles had relatively high nitrogen content and fungal biomass. Thus, selection of oviposition sites by stag beetles was likely to depend on both the log decay stage (or hardness) to protect immature stages from natural enemies and its nutritional properties to enhance the larval performance.

Keywords: oviposition, larval performance, nutrient, log, decay class

Introduction

The fate of offspring is often determined by their mother's decisions. For insects, this maternal dependence is very strong when larvae have limited mobility from the initial oviposition site in or near the food source and developmental environment of the larvae. This is especially the case for parasitoids and most xylophagous insects, where the safety and food supply of the immature stage inevitably depends on the maternal choice of oviposition site.

In order to maximise the fitness of their progeny, females must distinguish and competitively select high quality food sources within suitable environments for their progeny from among a range of variable resources, a trait which has become known as the "preference-performance" or "mother knows best" hypothesis (Jaenike, 1978). Larval performance can be experimentally evaluated through various traits, mostly larval or pupal weight, growth rate and survival rate (Singer et al., 1988; Craig et al., 1989; Hanks et al., 1993; Forister, 2004). Many factors, both the nature of those insects and surrounding environments, such as the life history, resource availability and selective pressure, have been reported to affect the maternal selection of oviposition sites and oviposition strategies (Scheirs et al., 2000; Mayhew, 2001; Saint-Germain et al., 2010).

The decomposition of dead wood is an essential process in ecosystems (including nutrient recycling) and in turn is an important natural resource in terrestrial ecosystems where it acts as a microhabitat and reservoir for many living communities, especially invertebrates and microorganisms (Yee et al., 2001; Kehler et al., 2004; Nordén et al., 2004; Lachat et al., 2012). Saproxylic insects are the dominant

invertebrates that utilise plant remains. Although wood contains a high portion of carbohydrate, its complex constituents are difficult to digest; as a consequence of its high carbon content, if provides a low level of essential elements for the growth of organisms (Schmidt and Czeschlik, 2006). Therefore, wood-feeding insects should have an oviposition selection strategy in order to optimise the growth and survivability for their progeny. Many factors have been reported to affect the occupation of wood materials by such insects. The types of deadwoods, such as standing dead trees and fallen logs, and the surrounding conditions, such as sun-exposed and shaded, have been shown to influence the species richness and numbers of individuals found within those wood remains (Jonsell and Weslien, 2003; Kappes and Topp, 2004). The level of decay of dead wood causes changes in its physical and nutritional properties and so provides different conditions that are suitable for different communities, including saproxylic insects, which often lead to a pattern of succession (Saint-Germain et al., 2007; Saint-Germain et al., 2010).

Stag beetles (Coleoptera: Lucanidae) are saproxylic insects, where the larval stages live in and feed on the decaying wood. Environmental factors, particularly nutrition obtained during the larval stage are a major influence on the adult body and weapon size (Gotoh et al., 2011; Gotoh et al., 2014). The feeding of stag beetle larvae, *Cyclommatus metallifer* Boisduval, 1835, with different amounts of food supply resulted in distinctive differences in the larval growth performance and adult morphology (Tanahashi et al., 2009; Gotoh et al., 2011; Tanahashi and Kubota, 2013). Weapon and body size of male stag beetles are important factors determining the outcome of fights. Males with relatively larger body size gain more fighting and mating success than smaller males (Okada and Hasegawa, 2005; Inoue and Hasegawa, 2013;

Goyens et al., 2015b; Mills et al., 2016). This indicates the importance of food during the larval stage as a major component of reproductive success in adults.

Due to the strong correlation between adult body size and the nutrients obtained during the larval stage, oviposition site selection of female stag beetles should rely on some properties of wood to improve the performance of their offspring and increase their fitness. However, knowledge about the relationship between occurrence of stag beetle larvae and properties of their food source is limited, especially for tropical species. This work aimed to investigate factors that influenced oviposition choice by female stag beetles in the dry-evergreen forest of south-eastern Thailand. Possible factors, both physical and nutritional properties, of the logs were examined in order to find the factor(s) relating to potential preference of stag beetles for selecting suitable decaying wood for oviposition.

Materials and Methods

Study Site

The study area was located at 12° 55′ N to 12° 59′ N and 102° 17' E to 102° 22' E covering an area of approximately 52 km^2 in the dry-evergreen forest of the Marine's Paramilitary Task Force, Thewa Pitak Camp, Pong Nam Ron district, Chanthaburi province, Thailand. Most parts of the area were rolling plains with an elevation range of 170–300 m above mean sea level. The forest was predominantly comprised of trees in the families Fabaceae, Dipterocarpaceae, Lythraceae and Tetramelaceae. The average annual rainfall over the 30-y period from 1981–2010 was 2,994.2 mm/y.

Sampling

Sampling was conducted every two months from July, 2013 to November, 2014. Two randomly selected areas, each of 0.25 km^2 size, were extensively surveyed for approximately five hours each time, except for the first survey (July, 2013) in which only one plot was surveyed. Any surveyed plot was then excluded from future surveys, resulting in each of 17 individual plots being surveyed once in the study period.

Fallen logs and coarse woody debris of more than 10 cm in diameter were examined for the presence of stag beetles by breaking them into small pieces. Soil underneath the logs was also observed for soil dwelling species. Stag beetles (larvae, pupae and adults) found inside or under the logs were collected and maintained in the laboratory. Determination of the decay stage of the log was adapted from Hautala et al. (2004) by observing the appearance of the wood characters combined with the iron fork (17 cm long and 0.8 cm diameter) probe test for wood hardness, with each log being classified into one of six decay classes (Table III-1). For logs that contained more than one decay class in the same log, the decay class was determined from the part which had immature stag beetles (if present), or from the highest decay class in the absence of stag beetles. In this study, preliminary observation indicated that stag beetles were never found in logs of decay class I, and so logs of this decay class were excluded from this study. Pieces of wood (approximately 150 g of wet weight) were collected and brought to the laboratory for the wood property analysis. For logs with stag beetles, wood for analysis was sampled from nearby to the larval galleries (within a radius of 20 cm) but not in an area that had been infested by larvae. For logs without stag beetles, wood was randomly collected from the site of the log with the highest decay stage.

Plant species of the logs could not be clarified because nearly all of the decaying logs had lost their species-specific characteristics used in their identification.

Table III-1 Characterization of logs into each decay class (adapted from Hautala et al., 2004)

Decay class	Description
I	Newly fallen log. Bark remaining intact. Wood is still very hard. Fork cannot stab or only a few millimeters into it.
II	Wood is hard. Bark slightly broken up. Fork can stab 0.5–1 cm into it.
III	Wood is quite soft. Bark losing > 50%. Fork can stab 1–3 cm into it.
IV	Wood is soft. Fork can stab 3–5 cm into it.
V	Wood is very soft, without bark. Fork can stab > 5 cm into it. It disintegrates easily between fingers.
VI	Most parts (> 50%) of wood are hollow or have many cavities (usually caused by termites).

Rearing and Identification

Stag beetle larvae were individually reared in small plastic cups (diameter 12 cm, height 7 cm) filled with 0.5 L artificial diet for larval weight < 15 g and in large plastic containers (diameter 23 cm, height 11 cm) filled with 4.5 L of diet for larval weight > 15 g to provide adequate diet to complete the development. Artificial diet was made from the fermented sawdust of the rubber tree, *Hevea brasiliensis* Mull. Arg., supplemented with 10% (w/w) wheat flour, 60 ± 5% (w/w) water content, pH 7.5–8.0, under constant darkness at 29 ± 4 ºC and 75 ± 5% relative humidity (modified from Ek-Amnuay, 2009). Stag beetles obtained as pupae were kept in the plastic cups laid with

moist tissue paper at the bottom and maintained at the same condition as the larval rearing. After adult emergence, they were identified to species based on their morphological characteristics using “The Lucanid Beetles of the World” (Mizunuma and Nagai, 1994), “Lucanidae of Thailand” (Pinratana and Maes, 2003) and “Beetles of Thailand” (Ek-Amnuay, 2008).

Breeding Experiment

Since development time of stag beetles can vary depending on the species, decay class of wood at the time of collection may shift from the time at oviposition. Therefore, breeding experiment was performed with the stag beetles collected from the field in order to roughly estimate their developmental time. Breeding method was modified from Ek-Amnuay (2009) using plastic boxes (28 × 40 × 25 cm) containing the fermented sawdust substrate filled for a half of the box and one or two moist decaying timbers (approximately 12 × 30 cm) collected from the field. Stag beetles were paired to mate and then females were placed into the breeding container. After 30 days, stag beetle larvae (mostly first instars) were collected and individually reared with the same condition as described in the rearing procedure. Instars were identified to stage from the capsule width according to the Dyar’s rule (Dyar, 1890).

Measurement of Wood Properties

All the logs with stag beetles (52 logs) were measured for their physical and nutritional properties, while 26 logs that did not contain beetles were also randomly selected for measurement.

Wood samples from each log were divided into three portions. The first portion was used for pH measurement. The fresh wood samples were ground and mixed with 2x (by weight) of distilled water, then the suspensions were measured by using pH meter (model pH 900, Precisa). The second portion was tested for other physical properties. Wood samples were weighed and then dried in a hot air oven at 60 °C until at constant weight, with the water content (% (w/w)) being calculated from the difference in the wet and dry weights. Wood density was estimated from dry weight divided by volume. Volume was estimated by water displacement. Water absorption capacity was measured by weighing water saturated wood (wood samples were immersed in distilled water for 3 days), and then calculated as milliliters of water held by 1 cm^3 of wood (Saint-Germain et al., 2007). For the last portion, dry wood samples were ground in a blender and sieved through a 30-mesh screen for nutritional analyses. Carbon (C) and nitrogen (N) contents in the wood were analysed using a CHN analyser (LECO Corporation, 628 Series: CHN) as described in Vose and Swank (1993). Soluble sugar content was measured by ethanol extraction followed by the phenol-sulfuric assay as described in Chow and Landhäusser (2004). Neutral detergent soluble (NDS) was analysed as described in Van Soest et al. (1991). Total phenol was measured by the Folin-Ciocalteu method as described in Hagerman et al. (2000). Fungal biomass was measured as the glucosamine-equivalent as described in Ramachandran et al. (2005).

Statistical Analyses

Preference of stag beetles in decaying logs was tested based on (1) the decay class of logs and (2) log and wood properties using principal component analysis (PCA). Multivariate analysis of variance (MANOVA) was used to assess whether the

decay class are exactly correlated with the wood hardness and/or other properties. Decay class VI logs were excluded from this analysis as termites may have entered the logs only recently. The ratio of occurrence and beetle number between different decay classes were analysed using Fisher's exact test. PCA was conducted with 11 log/wood properties using the prcomp function in R. Statistical analyses were performed using the *base*, *ggfortify* and *ggplot2* packages in R version 3.3.0 (R Development Core Team, 2016).

Results

Stag Beetle

In total, 255 stag beetles were collected in this study. Of these, 226 specimens were collected as immature beetles (larval or pupal stage), but only 139 specimens (61.5%) developed into the adult stage. These stag beetles were identified to eight species from five genera: *Aegus chelifer chelifer* MacLeay, 1819, *Prosopocoilus buddha* (Hope, 1842), *Prosopocoilus inquinatus nigripes* (Boileau, 1905), *Prosopocoilus jenkinsi* (Westwood, 1848), *Dorcus titanus* (Boisduval, 1835), *Odontolabis siva* (Hope & Westwood, 1845), *Nigidius* sp. 1 and *Nigidius* sp. 2. Of these, *A. chelifer chelifer* was the most abundant in terms of both occurrence and number of individuals (Figure III-1).

Logs containing more than one stag beetle species were found for nine logs, which consisted of three species for one log (*A. chelifer chelifer*, *D. titanus* and *P. buddha*) and two species for eight logs (*A. chelifer chelifer* and *Nigidius* sp. 1; *A.*

chelifer chelifer and *P. buddha*; *A. chelifer chelifer* and *P. inquinatus nigripes*; *A. chelifer chelifer* and *P. jenkinsi*; *D. titanus* and *P. inquinatus nigripes*).

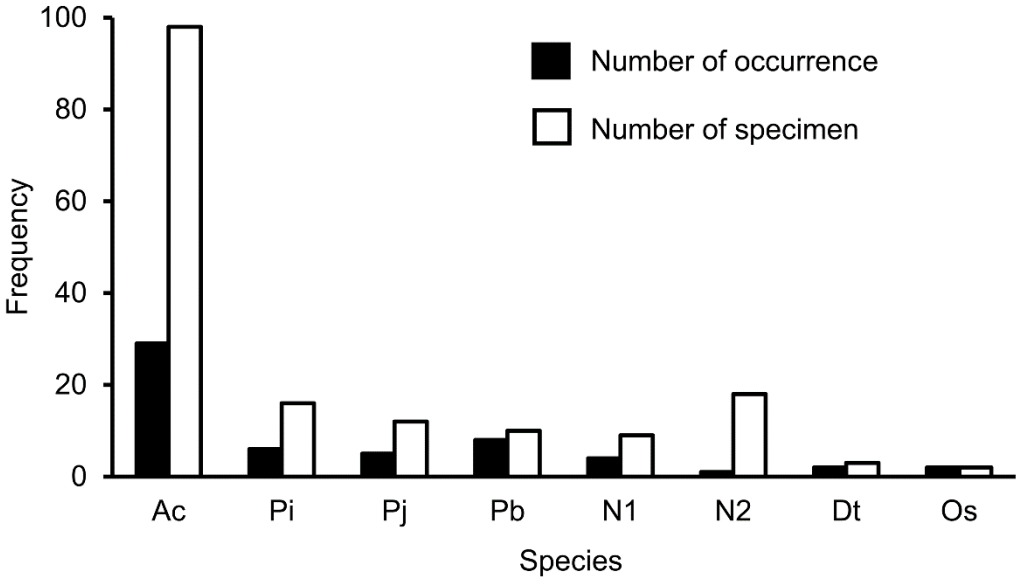

Figure III-1 Occurrence and number of specimens of each stag beetle species found in the study area. Ac = *A. chelifer chelifer*; Pi = *P. inquinatus nigripes*; Pj = *P. jenkinsi*; Pb = *P. buddha*; N1 = *Nigidius* sp. 1; N2 = *Nigidius* sp. 2; Dt = *D. titanus*; Os = *O. siva*.

Breeding

Breeding experiment was successful for seven out of eight species, except for *Nigidius* sp. 2 which the larvae were not obtained. Eggs and larvae could be found in both the supplied timbers and sawdust substrate for *A. chelifer chelifer*, *P. buddha*, *P. inquinatus nigripes* and *D. titanus*, only within the timbers for *P. jenkinsi*, and only in the sawdust substrate for *O. siva*. The final instar (3rd instar) was the longest developmental period during larval stage. Developmental time from egg to adult stages

varied among the species ranging from three months for *A. chelifer chelifer* to more than one year for *O. siva* (Table III-2).

Table III-2 Developmental time of each stag beetles species collected from the field

Species	Developmental time (day)			
	Before 3rd instar[a]	3rd instar	Pupa	Total
A. chelifer chelifer	40–50	25–50	15–20	80–120
P. buddha	50–60	180–250	20–30	250–340
P. inquinatus nigripes	50–60	180–220	20–30	250–310
P. jenkinsi	50–60	150–200	20–30	220–290
D. titanus	60–75	70–270	25–35	155–380
O. siva	60–75	250–450[b]	25–35[b]	335–560[b]
Nigidius sp. 1	35–45	120–150	10–15	165–210

Nigidius sp. 2 was excluded because breeding was not successful.

[a] The time was counted from the day that females were released into the breeding containers to the day of the end of 2nd instar.

[b] The data were from individuals which could pass to adult stage. Most larvae (> 90%) died when the age of 3rd instar was more than 450 days.

Decay Class

Logs were significantly different among decay classes (MANOVA: Pillai's Trace = 0.303; F = 2.450; df = 11, 62; $P = 0.013$) in their wood density (ANOVA: $P < 0.001$) and water content (ANOVA: $P = 0.021$) (Figure III-2). This indicated that the decay class could infer wood hardness.

In total, 270 logs were examined during the study. The most frequent decay class of logs (decay class I were excluded) was class III (94 logs), followed by class IV (61 logs), class II (48 logs), class V (35 logs) and class VI (32 logs). Of these, 52 logs (19.3%) were discovered to contain stag beetles (Figure 2.3A). The probability of stag beetle occurrence in each log decay class was significantly different ($P = 0.04$), with a high frequency in the moderate decay classes (class II–IV). The ratios of occurrence of each decay class, ranked from the highest to the lowest, were 27.9%, 21.3%, 18.8%, 14.3% and 3.1% for log decay class IV, III, II, V and VI, respectively.

The number of beetles found in the logs was highly varied, ranging from 1 to 51 individuals per log. The highest number of beetles were found in logs of decay class IV (104 larvae, 1 pupa and 2 adults) followed by class III (61 larvae, 2 pupae and 3 adults), II (35 larvae and 10 adults), V (14 larvae and 5 adults) and VI (9 larvae and 9 adults). *A. chelifer chelifer* stag beetles, which had the highest occurrence, were also tested. The ratios of occurrence in each decay class ranked from the highest to the lowest were 18.0%, 12.5%, 9.6%, 8.6% and 0% for log decay class IV, II, III, V and VI, respectively, but was not significantly different among decay classes ($P = 0.08$) (Figure III-3B).

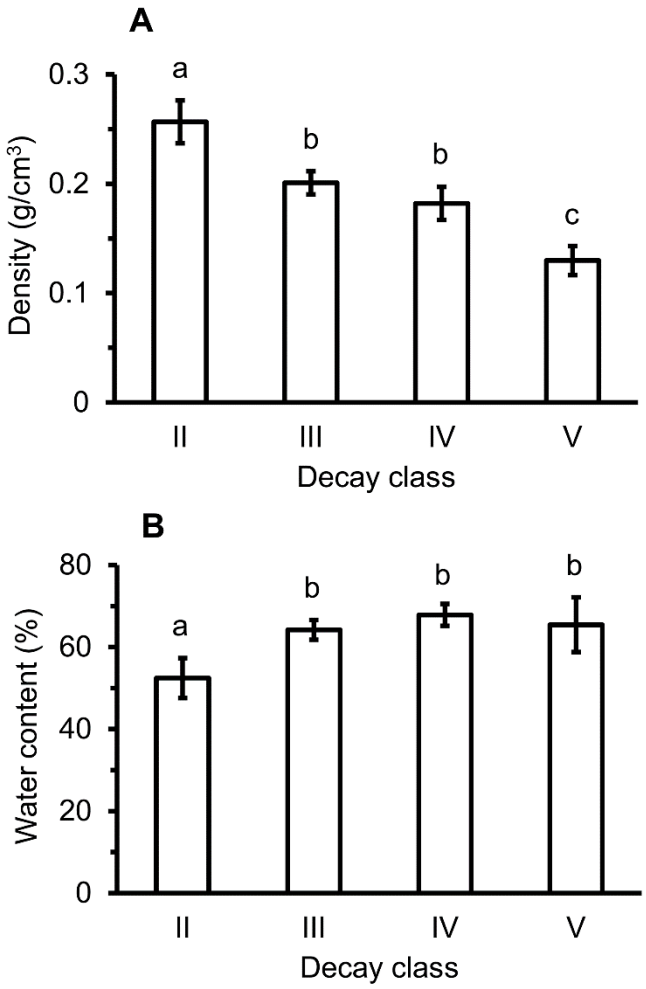

Figure III-2 Comparisons of (A) wood density and (B) water content of each decay class. Bars represent the mean (± SE) values. Significant differences are denoted by different letters above the column (ANOVA: $P < 0.05$).

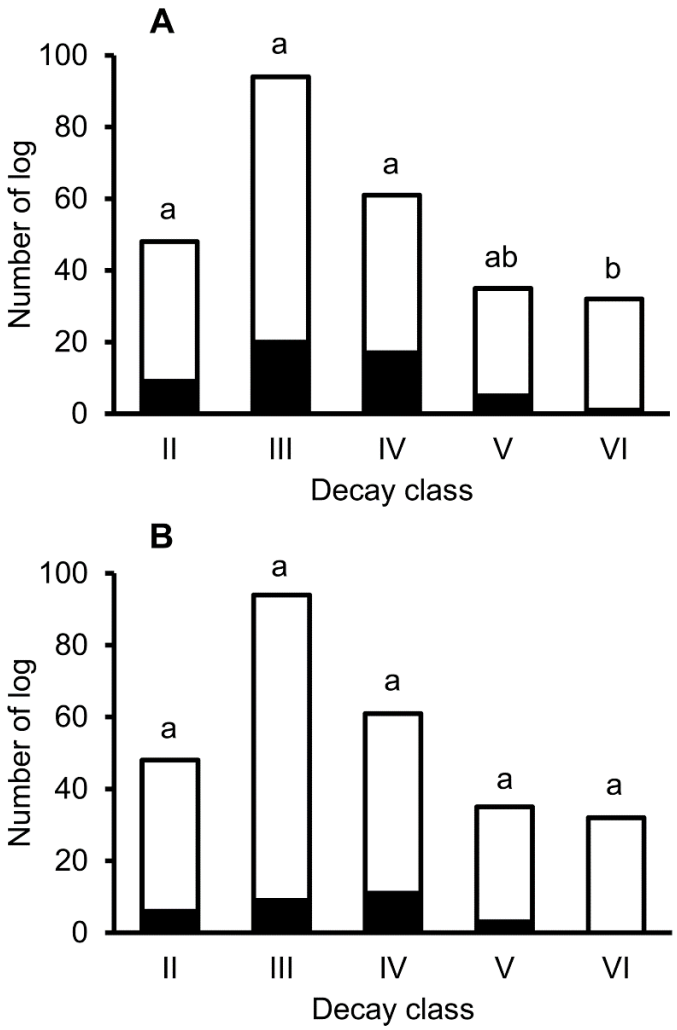

Figure III-3 Occurrence of stag beetles found in decaying logs of different decay classes. (A) Occurrence of logs with all stag beetles and (B) occurrence of logs with *A. chelifer chelifer*. Black and white boxes refer to number of log with and without stag beetles, respectively.

Wood Properties

PCA was conducted with 11 properties of decaying logs (n = 78). Eigenvectors of the first three principal components (PCs 1–3) are presented in Table II-3. Biplots of the first two PCs were constructed, which explained 54.55% of the total variance

(Figure III-4). PC1 was mainly related to water absorption capacity, water content, total phenol, NDS and soluble sugar content, while PC2 had high loading on diameter, fungal biomass, N content and C/N ratio.

The ordination of sample plots showed the features of decaying log selection by stag beetles. Most stag beetle occurrence was observed in logs characterised with high water absorption capacity, water content, fungal biomass and N content, and low C/N ratio, soluble sugar content and total phenol (Figure III-4A). Similar result was also obtained when only *A. chelifer chelifer* stag beetle occurrence was considered (Figure III-4B).

Table III-3 Factor loading on the first three principal components of physical and nutritional properties of decaying logs

Variable	PC1	PC2	PC3
Wood density	0.265	0.260	-0.355
Diameter	0.005	-0.336	0.280
Water content	-0.343	-0.215	0.387
Water absorption capacity	-0.353	-0.090	0.311
pH	-0.265	0.107	-0.470
Total phenol	0.464	0.022	0.112
NDS	0.319	0.200	0.325
Soluble sugar content	0.474	0.001	0.269
Fungal biomass	-0.123	0.487	0.218
N content	-0.117	0.509	0.292
C/N ratio	0.214	-0.466	0.292
Eigenvalue	3.407	2.593	1.282
% Variance	30.972	23.575	11.659
% Cumulative variance	30.972	54.547	66.206

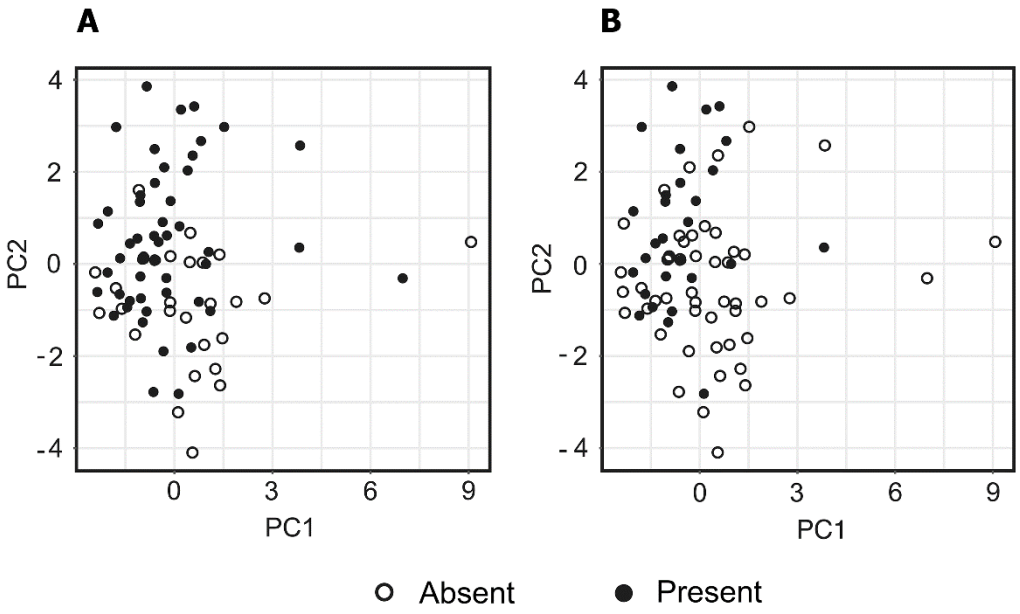

Figure III-4 Biplots of the first two principal components of (A) all stag beetle occurrence and (B) only *A. chelifer chelifer* occurrence based on 11 properties of decaying logs in the study area.

Discussion

The proportion of stag beetle occurrence in fallen logs in the study area was relatively low when compared with the total number of examined logs. This low level of colonization of decaying logs may relate to the high availability of logs in various decay stages within the forest. In addition, stag beetle larvae could live in the standing dead trees, underground logs, or even the dead portions of living trees (Wood et al., 1996). However, this study examined only aboveground fallen logs. Moreover, some stag beetles require specific conditions for growth, which might be collected but could not develop to adults under the rearing condition (Araya, 1993b, a; Ek-Amnuay, 2009). Thus, any inferred oviposition preference may not cover all stag beetle species in the

study site and/or oviposition sites and so the number of beetles and their occurrence may be underestimated for some species. Adults were not excluded from the analyses of the decaying wood preference because the presence of them in logs is mainly due to two reasons, (1) they just recently become adults and still live inside or underneath the logs, or (2) they come to the logs for oviposition (Harvey et al., 2011b). This study showed that the occurrence of stag beetles was highest in logs of a moderate decay class (classes II, III and IV), and decreased in logs of a more advanced decay (classes V and VI), while the PCA revealed that the occurrence was high in logs with high nutritional values (e.g. N content and fungal biomass). It should be noted that most samples used for PCA were from logs with stag beetles that resulted in incongruence between PCA and the preference based on the log decay class (wood hardness). Regardless, this indicated that the preference of stag beetles for oviposition in logs was likely to have a certain decay window and wood properties.

It was possible that the shift of the decay class could take place during stag beetles lived inside the logs, and thus affected to the estimation of the oviposition preference. Unfortunately, the age of the logs after the trees died and the decomposition rate could not be accurately estimated in this study due to many factors, such as plant species, diameter and environmental exposure. Nevertheless, many studies reported the time for complete decomposition of fallen logs ranged from several years to several decades (Weedon et al., 2009; Hérault et al., 2010; Freschet et al., 2012). By comparing with the data obtained from the breeding experiment, most species could complete development within one year, thus overestimation of the decay class relating to the oviposition preference should be negligible.

The results of this study indicated that the decay class, which was related to wood density or hardness, was the important factor related to the oviposition preference. The effect of the wood hardness on the performance of saproxylic insects has mostly been studied in termites, where wood with a high hardness (or density) resulted in a reduced ingestion rate in the termites and/or caused a loss of a large amount of energy being spent on feeding (Hochuli, 1996; Peralta et al., 2004). This could further decrease the insect performance due to providing insufficient nutrients in a given time. From the fact that wood typically contains a low quality of nutrients (Schmidt and Czeschlik, 2006), feeding on wood with a suitable softness would help intake food faster, and so obtain the nutrients at a sufficient rate (Bernays and Simpson, 1982). Another factor related to the decay level of logs was the water content, which increased with increasing log decay (higher decay classes). Generally, wood with a high decay class had a low density and high porosity, which allowed water to permeate between the wood tissues and remain inside the logs. Stag beetle larvae have soft bodies and an unsclerotised exoskeleton, and so the loss of water from their bodies is likely to happen quickly under a dry condition. Thus, logs with a moderate to advanced stage of decay could provide stag beetle larvae with sufficient water during their development until the adult stage.

One possible explanation for the low occurrence of beetle larvae in the advance decay logs may relate to the risk from natural enemies. Stag beetle larvae have soft bodies, short legs, limited mobility, and high nutritional values, and so they are susceptible to exposure to the external environment and are vulnerable to attack by predator. Although late decay stage wood provides a softer wood texture and potentially reduced lignocellulose content that aids ingestion and a higher water content, it allows

predators or parasites easier access to the eggs or larvae. Parasitic flies, parasitic wasps, mammals and birds have been reported to attack the stag beetles living inside logs (Ritcher, 1958; Wood et al., 1996). During the log-surveys in this study, some decaying logs were destroyed by wild animals, probably wild boars (based on the observed footprint), which have been reported to predate on larval and adult stages of stag beetles (Harvey et al., 2011a). Thus, living in decaying logs with an adequate hardness could reduce the risk from natural enemies better than in the softer more advanced decay logs.

PCA revealed that the occurrence of stag beetles was highly related to N content and fungal biomass. Among the nutrients available in plants to insects, nitrogen is one of the most important factors for growth and host preference in many herbivorous insects (Faeth et al., 1981; Minkenberg and Ottenheim, 1990; Huberty and Denno, 2006; Tanahashi et al., 2009), and this study supported the host preference based on the N content in wood. The low level of N in plants is often insufficient for insect growth (Ayres et al., 2000), where the low N and high C/N ratio are frequently the limiting factors for growth and development (Jönsson et al., 2004; Berner et al., 2005; Huberty and Denno, 2006; Zehnder and Hunter, 2009). In support of this for stag beetles, exogenously increased N content in the diet of stag beetle larvae was shown to increase their growth rate (Tanahashi et al., 2009).

Another factor related to the occurrence was fungi. Some logs with stag beetles showed the presence of dense visible mycelium inside the wood. Wood-decaying fungi have previously been shown to be associated with stag beetle larvae and were a factor that could attract female stag beetles into decaying logs. (Araya, 1993b, a; Tanahashi et al., 2009; Harvey et al., 2011b; Tanahashi and Kubota, 2013). Fungi has been

reported to increase the performance of stag beetle larvae, where some stag beetles, such as *Dorcus rectus* (Motschulsky, 1857), that are fungivorous and feed on the mycelia of wood-decay fungi alone have a higher growth than those that fed on diet without fungal mycelia (Tanahashi et al., 2009; Tanahashi and Kubota, 2013). Fungi normally play an important role in the nutritional ecology of saproxylic insects in several ways. Extracellular enzymes produced from the fungi breakdown the otherwise indigestible lignocellulose structure and soften the hard structure of wood making it softer and more readily ingested (Hanula, 1996), and some can also function inside the insect gut of some beetle families (Kukor et al., 1988). Structural polysaccharides in the wood are also converted to fungal biomass that can be used as a nutritional supplement for the beetles (Tanahashi and Kubota, 2013). Furthermore, the fungi can increase the ratio of N content, which is normally low in wood, by taking up N from the surrounding environment and concentrating it in their mycelia (Swift, 1977; Schmidt and Czeschlik, 2006). This could explain the high correlation between N content and fungal biomass in the PCA. However, it should be noted that not all stag beetle species require fungi for their growth. From rearing of stag beetles collected as larvae and breeding experiment, most of stag beetles could successfully develop without wood-decaying fungi in the diet. Wood-decaying fungi is, therefore, likely to be an option in oviposition preference for enhancing the growth of stag beetle larvae.

In summary, the occurrence of stag beetles was more frequently observed in logs of a moderate decay class and logs containing high nutrients (N content and fungal biomass). Advanced-decay logs could give benefits to the beetle larvae, providing more moisture and ease of ingestion, but at a higher risk from natural enemies. Early-decay logs could provide better safety, because of the hard structure of the wood, but less ease

of ingestion and a lower level of fungi and moisture. Preference for moderate-decay logs is, therefore, potentially the result of the balance between food qualities and risk from natural enemies. The oviposition preference of stag beetles might follow the preference-performance hypothesis by relying on both the survival rate of offspring and the growth performance. However, to confirm this assumption and provide a better understanding, further research is required.

CHAPTER IV

DIFFERENT ALLOMETRIC INTERCEPTS IN MAJOR *Aegus chelifer chelifer* STAG BEETLE MALES FROM URBAN AND FOREST HABITATS

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Abstract

Many insects, including stag beetles, possess exaggerated structures and these structures usually grow disproportionately with their body size. Allometry, i.e. the scaling relationship between such traits and other body parts (used as proxies of body size in a species), can provide valuable information about development and evolution. Males of *Aegus chelifer chelifer*, a stag beetle from forest and urban habitats in Thailand, were examined to clarify the allometric relationship between weapon (mandible) and body (elytra) size. The relationship between the log-transformed mandible and elytra length was non-linear and best fitted the piecewise linear model. Moreover, this model revealed the existence of dimorphism in males that could be divided into minor and major morphs based on their mandible size, in broad agreement with the morph classification based on mandible shape. Both morphs from the two populations (urban and forest) exhibited positive allometry, and the allometric slope declined in the major morph. Comparison between populations showed the allometric slopes were similar, but the intercepts were significantly different in the major morphs. The genetic or environmental basis should be further explored for the two morphs as well as any behavioural variation.

Keywords: population, piecewise model, mandible

Introduction

Allometry, or the scaling relationship between two body parts, provides valuable information in various fields of biology, including ontology, taxonomy and evolution (Eberhard and Gutierrez, 1991; Kawano, 1995, 2000; Knell et al., 2004). Many fighting beetles, such as rhinoceros, dung and stag beetles, have frequently been used as models for both static allometry (allometry of members at the same stage within the same population) and evolutionary allometry (allometry among populations or taxa) because of their shapes and weapon sizes (e.g. Eberhard and Gutierrez, 1991; Kawano, 1995; Emlen, 1996; Emlen and Nijhout, 2000; Kawano, 2000; Knell et al., 2004).

Males of stag beetles are larger than females and are normally equipped with large mandibles as a weapon to compete with rival males over females. Males also have a high intraspecific variation in body size in most species (Kawano, 2000; Harvey et al., 2011b; Hendriks, 2013). Moreover, their mandible varies in shape along the range of body size for many species, resulting in dimorphism or polyphenism in their mandible morphology (Kawano, 2000; Shiokawa and Iwahashi, 2000a; Iguchi, 2001, 2013; Mills et al., 2016; Romiti et al., 2016).

The size ratio between the weapon and body parts is an important factor in determining the fighting behaviour and reproductive strategy of some male beetles. Males of rhinoceros and dung beetles with a relative long weapon (major males) are more successful in male-male combat, while those with a smaller weapon (minor males) are less aggressive and use alternative strategies, such as a sneaking tactic, to access

females (Siva-Jothy, 1987; Emlen, 1997a; Karino et al., 2005). However, the scaling relationship between the weapon and body sizes of stag beetles uniquely depends on the species, and there is no general model or criterion to explain the allometry for all stag beetles (Kawano, 2000; Shiokawa and Iwahashi, 2000b; Romiti et al., 2016).

Therefore, the allometry between the mandible and body size (use elytra as proxy) was examined in males of *Aegus chelifer chelifer* MacLeay, 1819 (Coleoptera: Lucanidae), a small stag beetle that is widely distributed in the mainland of Southeast Asia (Mizunuma and Nagai, 1994; Pinratana and Maes, 2003; Ek-Amnuay, 2008) with a relatively short life cycle of approximately three to four months in captivity (Ek-Amnuay, 2009). Males are equipped with long, curved mandibles, while females have a smaller body size and shorter mandibles. Larvae develop inside and feed on decaying wood. Although *A. chelifer chelifer* have been frequently found in woodlands, but they have also been reported as one of a few species of stag beetles that are well-adapted to urban habitats with a limited food source (Ek-Amnuay, 2008, 2009). Thus, they act as an important saproxylic insect involved in deadwood decomposition and nutrient recycling in urban areas.

Here, the scaling relationship between the mandible and body size of male stag beetles collected from two habitats (urban and forest) was compared and classified the morph(s) based on their mandible shape and regression analysis. The allometry obtained from this study could provide fundamental knowledge in the scaling relationship and could be further applied to understand development, adaptation to local habitat and possible effects of sexual selection.

Materials and Methods

Specimens

Male *A. chelifer chelifer* were collected from urban and forest localities in 2012–2016. The identification to the subspecies was based on their morphological characteristics using "The lucanid beetles of the world" (Mizunuma and Nagai, 1994), "Lucanidae of Thailand" (Pinratana and Maes, 2003) and "Beetles of Thailand" (Ek-Amnuay, 2008). The urban population specimens were collected from Bangkok metropolitan area (Bangkok and Nonthaburi provinces; 13° 20' N to 14° 08' N and 100° 15' E to 100° 56' E), in the central plain of Thailand, while the forest population specimens were collected from tropical evergreen forest and dry-evergreen forest in Chanthaburi province (12° 18' N to 13° 20' N and 101° 41' E to 102° 32' E), south-eastern Thailand. To obtain a sufficient number of specimens for the analysis, the stag beetles collected from the field sites (n_{urban} = 36, n_{forest} = 55) using light traps, street lights and decaying logs were combined with museum specimens collected in 1937–2016 ($n_{urban/minor}$ = 4, $n_{urban/major}$ = 20, $n_{forest/minor}$ = 5, $n_{forest/major}$ = 20: morph classification based on visual inspection of the mandible morphology).

Measurement

Stag beetles were photographed from dorsal view using a digital camera (Olympus TG-4, Tokyo, Japan), while mandible length (ML) and elytra length (EL) were measured from the digital images to the nearest 0.1 mm using tpsDig2 software version 2.17 (Rohlf, 2013). The ML was measured as a straight line from the tip to the

base of the left mandible, while the EL was measured along the middle line from the base of the scutellum to the posterior end of the elytra.

Qualitative Classification

Adults of *A. chelifer chelifer* exhibit sexual dimorphism (Figure IV-1). Males are normally larger in size, with longer curved mandibles with blunt basal teeth located near clypeus, and shining elytra with distinctively longitudinal grooves. Females have an oval body shape, strong punctures on head and pronotum, dull elytra, and relatively short mandibles (Pinratana and Maes, 2003; Ek-Amnuay, 2008). Males of *A. chelifer chelifer* have been classified into two morphs on the basis of the visual inspection of their mandible shape. The major morph occurred in males which had large mandibles with median tooth on the inner margin of each mandible, while the minor morph lacked these characters (Figure IV-1).

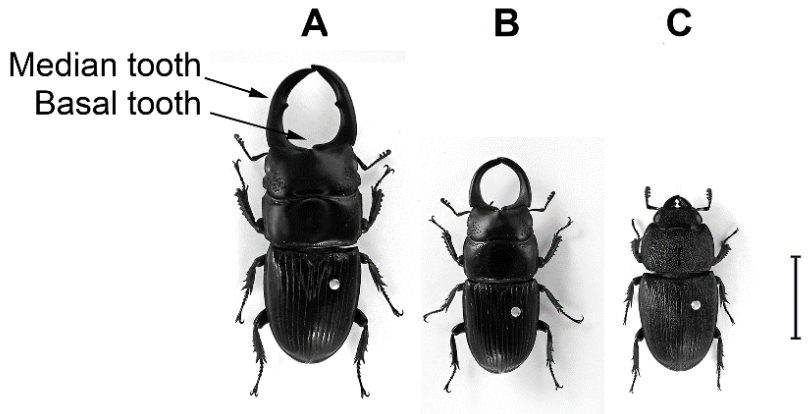

Figure IV-1 Comparison of the major and minor males, based on mandible shape, and female of *A. chelifer chelifer*. (A) Major male morph with a median tooth on the inner margin of each mandible. (B) Minor male, where the inner margin of the mandibles are smooth. (C) Female. Scale bar = 1 cm.

Allometry

In this study, ML and EL were used as a measure of weapon and body size, respectively. Other body parts, such as the head and pronotum, were excluded from the analysis due to ambiguity as to whether they should be considered as weapons or body parts. The allometric analysis followed the procedure suggested by Knell (2009). First, the natural log of ML was plotted against the natural log of EL, and then it was tested for linearity by fitting to the quadratic model shown in Eq. (4.1);

$$Y = \beta_0 + \beta_1 X + \beta_2 X^2 + \varepsilon, \quad (4.1)$$

where X is the natural log of EL, Y is the natural log of ML, β_0, β_1 and β_2 are regression coefficients, and ε is the error.

If β_2 is significantly different from zero, then the relationship is likely to be non-linear, and other possible statistical models (by visual inspection from the scatter plots) were tested using ordinary least squares (OLS) regression, with the following models:

Simple linear,

$$Y = \beta_0 + \beta_1 X + \varepsilon. \quad (4.2)$$

Continuous piecewise (Eberhard and Gutierrez, 1991),

$$Y = \beta_0 + \beta_1 X + \beta_2 (X - X^0) D + \varepsilon. \quad (4.3)$$

Discontinuous piecewise (Eberhard and Gutierrez, 1991),

$$Y = \beta_0 + \beta_1 X + \beta_2 (X - X^0) D + \beta_3 D + \varepsilon. \quad (4.4)$$

Alternative continuous piecewise (Kotiaho and Tomkins, 2001),

$$X = \beta_0 + \beta_1 Y + \beta_2 (Y - Y^0) D + \varepsilon. \quad (4.5)$$

Alternative discontinuous piecewise (Kotiaho and Tomkins, 2001),

$$X = \beta_0 + \beta_1 Y + \beta_2 (Y - Y^0) D + \beta_3 D + \varepsilon. \quad (4.6)$$

For the models of Eberhard and Gutierrez (1991) shown in Eqs. (4.3) and (4.4), X^0 is the break-point, $D = 0$ if $X < X^0$, $D = 1$ if $X \geq X^0$. For the models of Kotiaho and Tomkins (2001) shown in Eqs. (4.5) and (4.6), Y^0 is the break-point, $D = 0$ if $Y < Y^0$, $D = 1$ if $Y \geq Y^0$.

Statistical Analyses

The goodness of fit was estimated using Akaike information criterion (AIC) for model selection, where the model that gave the lowest AIC scores was deemed to be the best fit model for describing the allometric relationship (Knell, 2009). Agreement between the two methods of the qualitative classification and the allometric model with the lowest AIC score, was evaluated using Cohen's kappa statistic (k) to verify the allometry and morph classification (Cohen, 1960; Iguchi, 2013). Based upon Fleiss (1981), $k < 0.40$ is a poor agreement, $0.40 < k < 0.75$ is a fair to good agreement, and $k > 0.75$ is an excellent agreement. The allometry between populations for equality of allometric slopes and intercepts was also compared using analysis of covariance (ANCOVA). The interaction term between elytra length and population was removed from the model due to non-significant result. Statistical analyses were performed using

the *base*, *segmented* and *irr* packages in R version 3.3.0 (R Development Core Team, 2016).

Results

The scaling relationship between the ML and EL in male *A. chelifer chelifer* stag beetles (Figure IV-2) was found to be non-linear in both populations (Table IV-1). The alternative continuous piecewise model (Kotiaho and Tomkins, 2001) gave the lowest AIC score for both populations, and was accordingly considered to be the best fit model for this allometric relationship in males of *A. chelifer chelifer*. Furthermore, the males could be divided into two morphs based on the allometric slope. Major males had a ML equal to or longer than the break-point (5.1 and 6.1 mm for the urban and forest populations, respectively), while minor males had a ML shorter than the break-point (Figure IV-3).

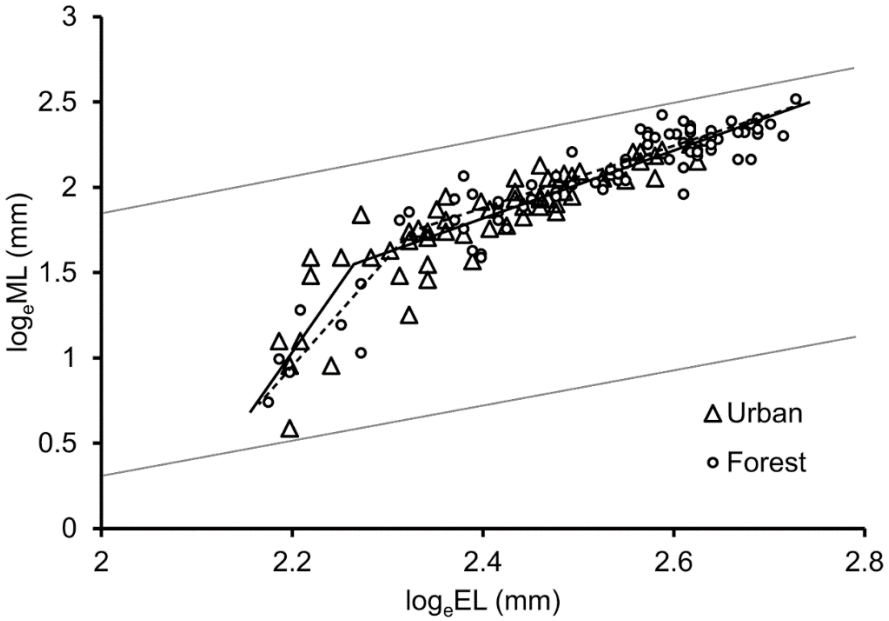

Figure IV-2 Scatter plot of the log-transformed mandible (ML) and elytra (EL) length of male *A. chelifer chelifer* collected from urban and forest habitats. The solid and dashed lines represent the regression lines of urban and forest populations, respectively. The grey parallel lines are isometry (slope = 1).

Table IV-1 Statistical model fitting analysis of allometry between the mandible and elytra lengths of male *A. chelifer chelifer* stag beetles

Population	Parameter	Estimate	SE	t	*P*	MSE	AIC	ΔAIC
Urban	Linear model							
	β_0	-4.388	0.475	-9.240	< 0.001	0.029	-35.05	134.85
	β_1	2.568	0.197	13.040	< 0.001			
	Quadratic model							
	β_0	-32.293	8.372	-3.857	< 0.001	0.025	-43.76	126.14
	β_1	25.878	6.986	3.704	< 0.001			
	β_2	-4.856	1.455	-3.338	0.001			
	Continuous piecewise model (Eberhard and Gutierrez, 1991) X^0 = 2.221, SE = 0.007							
	β_0	-39.219	7.706	-5.090	< 0.001	0.022	-51.48	118.42
	β_1	18.299	3.479	5.259	< 0.001			
	β_2	-16.164	3.571	-4.527	< 0.001			
	Discontinuous piecewise model (Eberhard and Gutierrez, 1991) X^0 = 2.221, SE = 0.007							
	β_0	-42.311	11.242	-3.764	< 0.001	0.022	-49.63	120.27
	β_1	19.708	5.100	3.865	< 0.001			
	β_2	-17.540	5.104	-3.437	0.001			
	β_3	-0.044	0.116	-0.380	0.705			
	Alternative continuous piecewise (Kotiaho and Tomkins, 2001) Y^0 = 1.636, SE = 0.067							
	β_0	2.011	0.064	31.427	< 0.001	0.003	-169.90	0
	β_1	0.205	0.041	5.010	< 0.001			
	β_2	0.138	0.056	2.461	0.017			
	Alternative discontinuous piecewise (Kotiaho and Tomkins, 2001) Y^0 = 1.636, SE = 0.067							
	β_0	2.011	0.067	29.789	<0.001	0.003	-167.90	2
	β_1	0.205	0.046	4.402	0.001			
	β_2	0.138	0.058	2.380	0.021			
	β_3	-0.0001	0.029	-0.004	0.996			

Table IV-1 (Continued)

Population	Parameter	Estimate	SE	t	*P*	MSE	AIC	ΔAIC
Forest	Linear model							
	β_0	-4.022	0.312	-12.890	< 0.001	0.023	-69.55	167.54
	β_1	2.401	0.123	19.460	< 0.001			
	Quadratic model							
	β_0	-30.179	4.097	-7.367	< 0.001	0.015	-101.65	135.44
	β_1	23.669	3.326	7.116	< 0.001			
	β_2	-4.307	0.673	-6.397	< 0.001			
	Continuous piecewise model (Eberhard and Gutierrez, 1991) X^0 = 2.324, SE = 0.020							
	β_0	-12.128	1.156	-10.495	< 0.001	0.014	-108.51	128.58
	β_1	5.958	0.505	11.799	< 0.001			
	β_2	-4.170	0.581	-7.175	<0.001			
	Discontinuous piecewise model (Eberhard and Gutierrez, 1991) X^0 = 2.324, SE = 0.020							
	β_0	12.622	1.731	-7.290	< 0.001	0.014	-106.66	130.43
	β_1	6.182	0.771	8.014	< 0.001			
	β_2	-4.371	0.784	-5.573	< 0.001			
	β_3	-0.031	0.082	-0.385	0.701			
	Alternative continuous piecewise (Kotiaho and Tomkins, 2001) Y^0 = 1.816, SE = 0.100							
	β_0	1.962	0.050	36.336	< 0.001	0.003	-237.09	0
	β_1	0.249	0.030	8.369	< 0.001			
	β_2	0.189	0.048	3.894	< 0.001			
	Alternative discontinuous piecewise (Kotiaho and Tomkins, 2001) Y^0 = 1.816, SE = 0.100							
	β_0	1.955	0.054	36.299	< 0.001	0.003	-235.25	1.84
	β_1	0.256	0.035	7.222	< 0.001			
	β_2	0.192	0.049	3.887	<0.001			
	β_3	-0.010	0.026	-0.390	0.698			

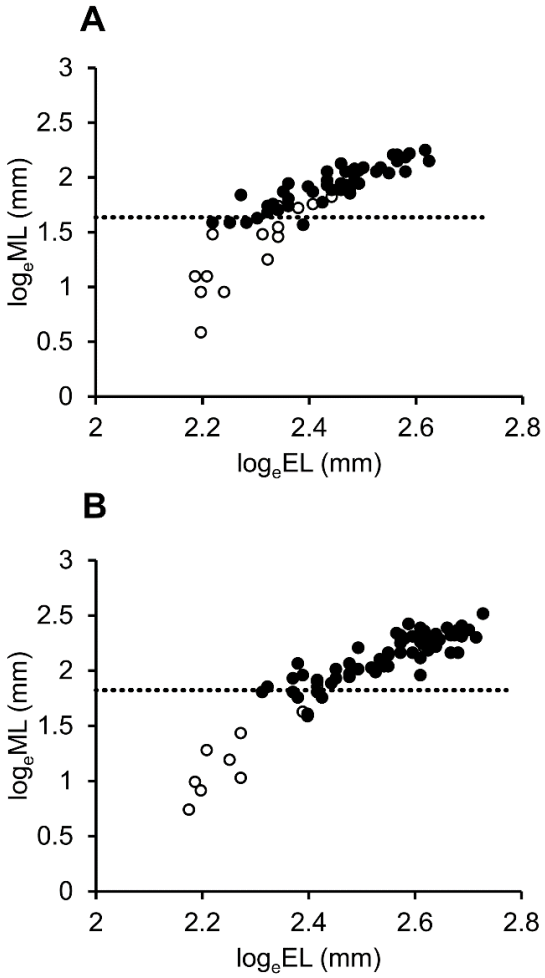

Figure IV-3 Morph classification based on mandible shape and the change of allometric slope in male *A. chelifer chelifer* beetles from the (A) urban and (B) forest populations. The dashed line represents the break-point of the allometric slope based on mandible length (ML; the alternative piecewise model) that separate minor males (with mandibles shorter than the break-point) from major males (with mandibles equal to or longer than the break-point) morphs. White and black circles refer to the minor and major males, respectively, by morphological classification based on their mandible shape.

From Figure IV-3, there are some borderline cases of disagreement between the two methods (mandible shape *vs*. allometric slope) for minor and major morph classification, but in most cases they are congruent. Statistically, Cohen's kappa statistic showed a fair to good agreement between the two methods of morph classification in both populations ($k = 0.591$ and $k = 0.576$, both $P < 0.001$ for the urban and forest populations, respectively). This result supported the use of the alternative piecewise model (Kotiaho and Tomkins, 2001) to explain the allometric relationship in these stag beetles.

Since the allometry fitted a non-linear relationship and was comprised of two allometric slopes, the minor and major morphs were separately compared the allometry between the populations. The allometric slopes of both the minor and major males between the two populations were not significantly different (Table IV-2), but the intercepts were significantly different only in the major males (Table IV-3).

Table IV-2 Analyses of covariance of the allometric slopes between two populations (urban and forest) of *A. chelifer chelifer* stag beetles

Variation	df	Mean square	*F*	*P*
Minor males				
$\log_e$(EL)	1	2.026	39.715	< 0.001
Population	1	0.013	0.264	0.611
Population × $\log_e$(EL)	1	0.016	0.322	0.575
Residual	26	0.051		
Major males				
$\log_e$(EL)	1	3.331	423.410	< 0.001
Population	1	0.046	5.881	0.017
Population × $\log_e$(EL)	1	0.003	0.323	0.571
Residual	106	0.008		

Table IV-3 Analyses of covariance of the allometric intercepts between two populations (urban and forest) of *A. chelifer chelifer* stag beetles

Variation	df	Mean square	*F*	*P*
Minor males				
$\log_e$(EL)	1	2.026	40.733	< 0.001
Population	1	0.013	0.271	0.607
Residual	27	0.050		
Major males				
$\log_e$(EL)	1	3.331	426.106	< 0.001
Population	1	0.046	5.919	0.017
Residual	107	0.008		

Discussion

Generally, the size relationship between two body parts, or allometry, can be expressed as a power law equation (Huxley, 1932), $Y = aX^b$, or as a simple log-transformed linear equation, $\log(Y) = \log(a) + b\log(X)$, where log(a) is the intercept at the *Y* axis and b is the allometric slope. However, allometry of many insects show a deviation from linearity, even after log-transformation of the data, which indicates that such traits irregularly increase with body size (Emlen, 1996, 1997a; Knell et al., 2004; Iguchi, 2013). In this study, the scaling relationship between the mandible and body (as elytra) size of male *A. chelifer chelifer* beetles best fitted the alternative continuous piecewise model (Kotiaho and Tomkins, 2001), and separated males into two morphs, major and minor, based on the change of the allometric slope. Normally, morphometric polymorphism in a species is visually determined or else is determined by plotting the frequency distribution of the body size (or an interesting trait) found in a population (Iguchi, 1998; Moczek and Emlen, 1999). These methods are suitable when the differences between morphs are easily distinguishable and a large number of specimens can be collected. In this study on male *A. chelifer chelifer*, the scaling relationship obtained by the regression analysis conformed well (but not totally) to the morph discrimination by mandible shape inspection, indicating that the use of this allometric relationship was likely to be broadly reliable for this species.

As expected, the mandible-body size (ML-EL) relationship showed a positive allometry (slope > 1), as found in other animals with sexually selected traits. However, the allometric slope declined in the major morph relative to that in the minor morph. The declining allometric slope in large males of *A. chelifer chelifer* reported here is

similar to that reported previously for some rhinoceros and stag beetles (Knell et al., 2004; Pomfret and Knell, 2006; McCullough et al., 2015). This phenomenon could limit the weapon size in very large individuals, and is believed to be caused by resource allocation during development (Knell et al., 2004; McCullough et al., 2015). For a brief time before moulting to pupae, insect larvae will arrest feeding to develop the adult structures. Because nutrients are no longer obtained during this stage, resource allocation to each body part then takes place. This effect would be stronger in large individuals due to higher cost for weapon production, and so the decreasing allometric slope represents a resource allocation trade off (Knell et al., 2004; McCullough et al., 2015).

The allometric slope was not significantly different between the urban and forest populations, but a significant difference in the intercept of major males at the break-point of the allometric slope was evident. Field studies and laboratory experiments in some animals, including stag beetles, have indicated that allometry can be altered by developmental conditions and from non-random mating (Emlen, 1996, 1997b; Harvey et al., 2011a; Egset et al., 2012). Although the stag beetles in this study were from different localities, the allometry was still similar. Nevertheless, it should be noted that the collected specimens were obtained from different generations, from various collecting techniques, and so from various microhabitats. Because static allometry is a population parameter, growth under various environmental conditions can cause individual variation in the weapon-body size relationship. The combination of specimens from the same population may then mask differences but rather yield a mean allometry similar to another population, as found in this study. Perhaps, breeding under controlled conditions is needed to confirm the allometric difference.

Although the morph classification obtained from the regression analysis of the ML-EL mostly agreed with the visual inspection based on the mandible shape, it may not reflect the life history trait dependent changes in these beetles. Studies in other beetles have shown that there were differences in fighting and reproductive strategies among different morphs. For example, minor males of the dung beetle, *Onthophagus acuminatus* Harold, avoided facing to (fighting) other males but rather used a sneaking strategy to access females (Emlen, 1997a). To ascertain if this is the case in *A. chelifer chelifer*, behavioural studies are needed to clarify the existence of male dimorphism in this species.

CHAPTER V

THE LARVAL PERFORMANCE AND ADULT BODY SIZE DIFFERENCES BETWEEN STAG BEETLE *Aegus chelifer chelifer* (COLEOPTERA: LUCANIDAE) POPULATIONS

Abstract

Stag beetles usually have great intraspecific variation in body sizes which can be affected by both environmental and genetic factors. However, direct study on wild specimens may be insufficient to clarify such variation due to confounding effects of ecological variance in natural habitats. Stag beetle *Aegus chelifer chelifer* MacLeay, 1819 from two localities, Bangkok metropolitan area and Chanthaburi province, Thailand, were reared under the same condition to investigate the differences in morphological characteristics between wild and captive breeding beetles and between populations. Narrow-sense heritabilities (h^2) of the observed traits in adults were not significant. Body size distribution of breeding specimens was less than wild specimens and the overlap of the body size distribution between populations was lower in the breeding beetles. Body size of Chanthaburi population stag beetles was significantly larger than Bangkok population. Allometries were also significantly different between populations, with respect to both allometric slopes and intercepts. The larval performances showed similar relative growth rate, but male stag beetles of Chanthaburi population had a longer feeding period, therefore larger body size in the adults. The

differences between the two populations could be explained by adaptation through larval performances and body size in order to respond to their habitats.

Keywords: habitat; urban; variation; adaptation; resource availability

Introduction

Many beetles, such as rhinoceros beetle, dung beetle and stag beetle, exhibit intraspecific variations in body size and their secondary sexual traits (Shiokawa and Iwahashi, 2000a; Kawano, 2002; Moczek, 2002; Kawano, 2003; Harvey et al., 2011a; Iguchi, 2013). Morphological variations of the adults are the result of physiological responses affected by external environment during larval stage, e.g. nutrition (Moczek, 1998; Shafiei et al., 2001; Moczek, 2002; Karino et al., 2004; Gotoh et al., 2011; Gotoh et al., 2014), latitude (Romiti et al., 2017), season (Hardersen et al., 2011) and larval density (Okada and Miyatake, 2010). These variations are important for sexual selection in male beetles. In rhinoceros beetles, body size and horn length were reported as crucial components to determine the outcome of fight between males, including their fighting and mating behaviours (Siva-Jothy, 1987; Karino et al., 2005; Okada and Hasegawa, 2005; Harvey and Gange, 2006; Inoue and Hasegawa, 2013).

Male stag beetles of many species have great variations in body size and mandible length. The largest males can have the body size up to nearly three times of the smallest males (Harvey and Gange, 2006; Harvey et al., 2011a). Morphological variations are not restricted only within a population, but variations across populations were also detected. Comparisons between two widely distributed European stag beetles, *Lucanus cervus* (L., 1758) and *Dorcus parallelipipedus* (L., 1758), showed that the

adult stag beetles collected from different habitats or countries had differences in the average body size (Harvey and Gange, 2006; Harvey et al., 2011a; Hendriks, 2013). Food type, food supply, and climate in each habitat have been suggested to cause the size variation which, in turn, affected the larval performances, such as duration of larval stages, and consequently affected the adult morphology (Harvey and Gange, 2006; Harvey et al., 2011a; Hendriks, 2013).

Environmental conditions are often heterogeneous in a habitat and also differ among localities that can result in phenotypic variations in insects within and among populations (Robertson, 1987; Harvey and Gange, 2006; Harvey et al., 2011a; Romiti et al., 2017). Moreover, phenotype due to some genetic effects can be masked by confounding effects from environmental variance in natural habitats (Robertson, 1987; Tsuchiya et al., 2012). It is interesting how morphological variations in a population will be changed from those observed in the wild if stag beetles are reared under the same environmental condition. Unfortunately, study about such variations of stag beetles in captivity are rarely documented, especially for tropical species.

To examine this variation, the experiment was conducted using stag beetle *Aegus chelifer chelifer* MacLeay, 1819, a tropical stag beetle widely distributed in the mainland of Southeast Asia in many habitats, such as forest and urban areas (Mizunuma and Nagai, 1994; Pinratana and Maes, 2003; Ek-Amnuay, 2008). A previous study in wild specimens revealed that allometry of *A. chelifer chelifer* between urban and forest populations was different, but the factor influences such difference is still unclear (Songvorawit et al., 2017b). Stag beetles were collected from two localities of Thailand. They were bred and reared under the same condition until adults. If body size of wild

stag beetles is primarily environmentally based, body size variation of stag beetles from captive breeding should be lower than those of the wild specimens and other phenotypic traits of the two populations should be similar. Study by rearing stag beetles in captivity can give valuable information on the importance of environmental factors to morphological variations.

Materials and Methods

Sources of Stag Beetles

Two populations of *A. chelifer chelifer* stag beetles were collected from Bangkok metropolitan area (Bangkok and Nonthaburi provinces; 13° 20' N to 14° 08' N and 100° 15' E to 100° 56' E) and Chanthaburi province (12° 18' N to 13° 20' N and 101° 41' E to 102° 32' E), Thailand, during 2012–2016. The distance between these two localities was approximately 200 km (Figure V-1). Collecting sites of Bangkok metropolitan area were urban areas, such as public parks and backyards. Climate is tropical savanna with average temperature of 24.9–33.3 °C and average rain fall of 1,648.2 mm/year (30-year average weather during 1981–2010, Thai Meteorological Department). Collecting sites of Chanthaburi province were forest areas. Climate is tropical monsoon with average temperature ranging from 23.8–32.2 °C and average annual rain fall of 2,994.2 mm/year (30-year average weather during 1981–2010, Thai Meteorological Department).

Wild-caught stag beetles were collected using light traps, street lights and decaying logs ($n_{Bangkok\text{-}male} = 36$, $n_{Bangkok\text{-}female} = 21$, $n_{Chanthaburi\text{-}male} = 55$, $n_{Chanthaburi\text{-}female} = 49$). Museum specimens collected in 1937–2016 from the same localities were also

included for the study ($n_{Bangkok\text{-}male}$ = 24, $n_{Bangkok\text{-}female}$ = 18, $n_{Chanthaburi\text{-}male}$ = 25, $n_{Chanthaburi\text{-}female}$ = 5). Identification at subspecies level was based on their morphological characteristics using "The Lucanid Beetles of the World" (Mizunuma and Nagai, 1994), "Lucanidae of Thailand" (Pinratana and Maes, 2003) and "Beetles of Thailand" (Ek-Amnuay, 2008).

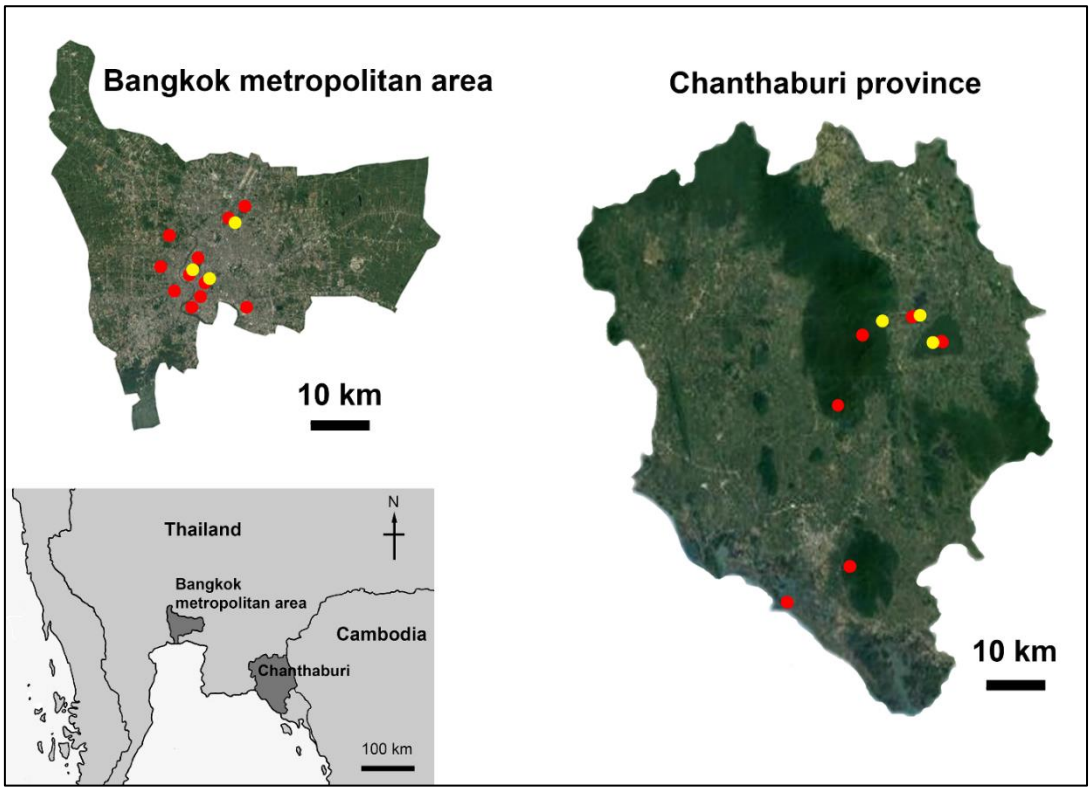

Figure V-1 Collecting sites of *A. chelifer chelifer* stag beetles, Bangkok metropolitan area and Chanthaburi province, Thailand. Red circles refer to collecting sites of beetle specimens and yellow circles refer to collecting sites of specimens which were used as parental generation for breeding experiment.

Breeding and Rearing

Live specimens were maintained in 200 ml plastic cups containing moist tissue paper under 12:12 dark/light period, at 29 ± 4 ºC and fed *ad libitum* with a piece of ripe banana (approximately 15 g) which was replaced every five days. Wild-caught beetles (collected during 2012–2014) were used as initial parental generation (F_0 generation) by random mating within the same population to make beetle stocks and to minimise the confounding environmental effects from their natural habitats.

For breeding experiment, stag beetles (F_1 or F_2 generations) were randomly selected from the stocks and paired within the same population to mate. Then, the mated females were transferred to the breeding boxes containing 4,500 ml sawdust substrate made from fermented sawdust of the rubber trees supplemented with 10% (w/w) wheat flour, 60 ± 5% (w/w) water content, pH 7.5−8.0, under constant darkness at 29 ± 4 ºC and 75 ± 5% relative humidity (modified from Ek-Amnuay, 2009). After late second instars (estimated from the head capsule width between 2.2–3.5 mm and weight higher than 0.2 g) were obtained, they were reared individually in 500 ml plastic cups fully filled with 220 g (wet weight) of the fermented sawdust substrate under the aforementioned conditions. Unfortunately, from total 20 breeding pairs of each population, beetle larvae were obtained from only 10 and 9 pairs from Bangkok and Chanthaburi populations, respectively.

Measurement of Larval Growth

Sex of stag beetle larvae can be identified from the presence of yellow ovoid shape of ovaries in females which are visible through larval cuticle at the dorsal part of

abdomen, while males lack this characteristic (Fremlin and Hendriks, 2014). Larval head capsule width was measured using a digital vernier caliper (Carbon Fiber Composites, Eagle One, Thailand) with the nearest 0.1 mm and larval weight using digital balance (OHAUS Adventure AR3130, New Jersey, USA) with the nearest 0.001 g every five days. Maximal larval weights of the second and third instars were assumed that they were the highest weights which were measured during the experiment. Feeding period of the third instar was counted from the first date when the larvae were found to reach the third instar to the first date when they were found to reach prepupal stage (identified from weight decrease, turning of cuticle from clear to more turbid and beginning of cocoon construction). Relative growth rate (RGR) was calculated from the weight change in 15 days during the early stage of the third instar, which is exponential growth, shown in Eq. (5.1);

$$RGR = (\ln W_2 - \ln W_1) / t \quad (5.1)$$

where W_1 is the weight at the first date of measurement at the third instar, W_2 is the weight after 15 days and t is the duration between measurement of W_1 and W_2 (15 days).

Since prepupal and pupal stages were susceptible to disturbance that could lead to death or abnormal development, their weight were checked until the beginning of prepupal stage, and then left for 45 days to ensure successful adult emergence. Larvae which died before prepupal stage were excluded from the analyses.

Measurement of Adult Morphology

Adult specimens were photographed from dorsal view using a digital camera (Olympus TG-4, Tokyo, Japan). Each body part was separately measured from the

digital images to the nearest 0.1 mm using tpsDig2 software version 2.17 (Rohlf, 2013). Mandible length (ML) was a straight line from the tip to the base of left mandible and elytra length (EL, used as proxy of adult body size) was the middle line from the base of the scutellum to the posterior end of the elytra).

Body Size Distribution

Inequality of body size (EL) distribution was examined following the methods described by Harvey and Gange (2006) and Magura et al. (2006). Initially, Lorenz curves were constructed. If all individuals in a sample group have the same size, the Lorenz curve is a straight diagonal line or line of equality. Then Gini coefficient (*G*), which represents deviation from equality, was quantified. *G* ranges from 0, when every individual has the same size, to 1 when, every individual except one has a size of zero (Damgaard and Weiner, 2000). Lorenz asymmetry coefficient (*S*) was calculated to examine which size classes contributed most to the total amount of inequality of a sample group. *S* is equal to 1 when the Lorenz curve is symmetric, *S* is less than 1 when the inequality is primarily due to relatively large number of small individuals, and *S* is greater than 1 when the inequality is primarily due to large individuals (Damgaard and Weiner, 2000).

Allometry

Allometry between ML and EL of males was examined. From previous study in wild-caught specimens of *A. chelifer chelifer* which were the same specimens as used in this study (Songvorawit et al., 2017b), allometric relationship between natural log of

EL and natural log of ML was best fitted continuous piecewise model proposed by Kotiaho and Tomkins (2001) as shown in Eq. (5.2);

$$X = \beta_0 + \beta_1 Y + \beta_2 (Y - Y^0) D + \varepsilon \quad (5.2)$$

where X is the natural log of EL, Y is the natural log of ML, Y^0 is the break-point, D = 0 if $Y < Y^0$, D = 1 if $Y \geq Y^0$, β_0 is the intercept, β_1 and β_2 are regression coefficients, and ε is the error.

From this model, the allometric relationship consisted of two linear slopes, and hence males could be divided into minor and major morphs based on the difference of such allometric slopes (Figure V-2). Unfortunately, minor males (males with mandible length shorter than the break-point of the regression line; 5.1 mm for Bangkok population, and 6.1 mm for Chanthaburi population) were obtained from the breeding with very low number ($n_{\text{Bangkok-minor male}} = 7$, $n_{\text{Chanthaburi-minor male}} = 1$). Thus, only major males (males with mandible length equal to or longer than the break-point of the regression line) were analysed by fitting to a simple linear model as shown in Eq. (5.3) using ordinary least squares (OLS) regression;

$$Y = \beta_0 + \beta_1 X + \varepsilon \quad (5.3)$$

where X is the natural log of EL, Y is the natural log of ML, β_0 is the intercept, β_1 is the regression coefficient, and ε is the error.

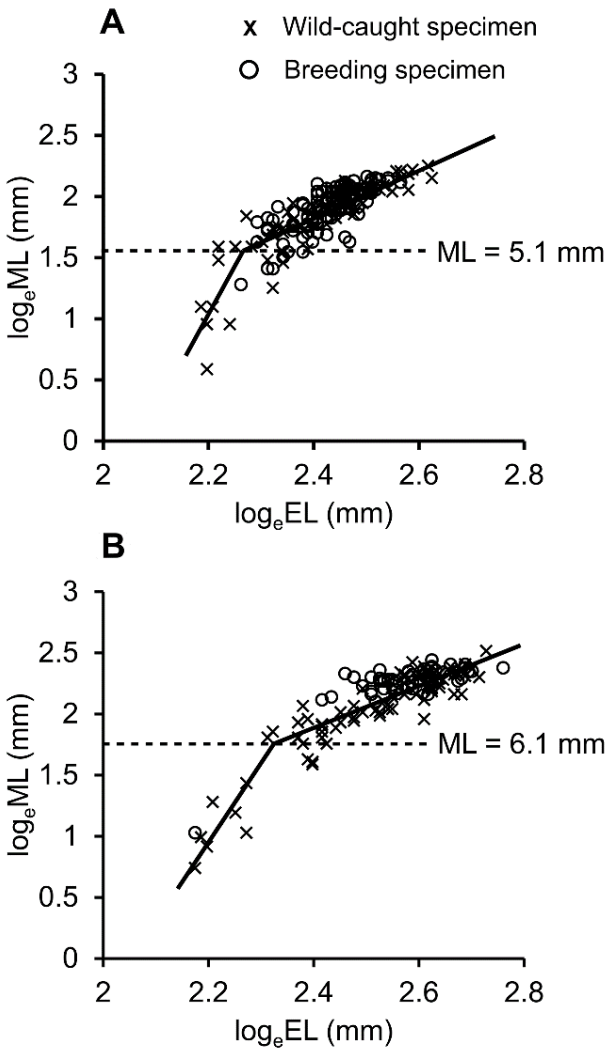

Figure V-2 Scatter plots of the log-transformed mandible (ML) and elytra (EL) length of male *A. chelifer chelifer* of (A) Bangkok population and (B) Chanthaburi population. The solid and dashed lines represent the regression lines of the allometric relationships and the break-points of the allometric slopes based on mandible length of wild-caught specimens, respectively.

Heritability

Narrow-sense heritabilities (h^2) of ML (only males) and EL were estimated by parent-offspring regression. Firstly, natural log of sire's (or dam's) body part was plotted against average son's (or daughter's) body part. Then, regression line was constructed and the slope was calculated. Narrow-sense heritability was estimated by twice of the regression slope (Falconer, 1989).

Statistical Analyses

Nonparametric statistics were used due to non-normal distribution of the data set (Shapiro-Wilk test, $P < 0.05$). Adult body size and larval performances were compared between populations by Wilcoxon rank sum test. To ensure whether larval weight could affect the adult size, the relationship between maximal larval weight at the third instar and EL was analysed by Spearman's rank correlation. Allometry of breeding specimens between the two beetle populations was compared by testing for equality of allometric slopes and intercepts using analysis of covariance (ANCOVA). All statistical analyses were conducted using *base* and *ineq* packages in R version 3.3.0 (R Development Core Team, 2016).

Results

Larval Performances

Results from rearing of stag beetle larvae indicated that most larval performances were significantly different between populations (Table V-1). Head capsule width and maximal larval weight were significantly greater in the Chanthaburi population for both sexes. Feeding period of the third instar of the Chanthaburi

population was significantly longer in males but shorter in females when compared to the Bangkok population. The RGRs of both populations were not significantly different. Maximal larval weight at the third instar positively correlated with EL of adults (Figure V-3).

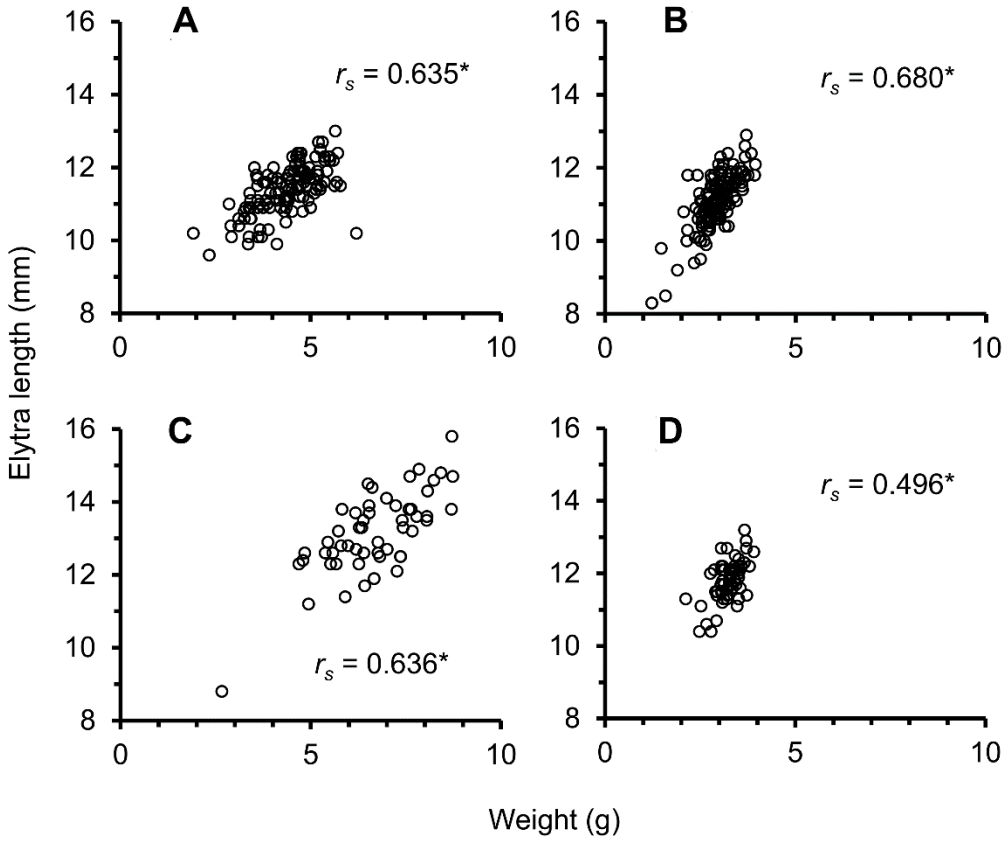

Figure V-3 Correlations between maximal larval weight at the third instar and elytra length of adults, (A) males of Bangkok population (n = 124), (B) females of Bangkok population (n = 135), (C) males of Chanthaburi population (n = 53), and (D) females of Chanthaburi population (n = 58). * indicates the correlation is significant at $P < 0.001$.

Table V-1 Comparisons of larval performances of *A. chelifer chelifer* stag beetles from breeding between Bangkok and Chanthaburi populations

Properties	Bangkok	Chanthaburi	W	*P*
Male				
Number of specimens	127	60		
Head capsule width of 2nd instar (mm)	3.0	3.3	769	< 0.001
Head capsule width of 3rd instar (mm)	6.2	7.0	610	< 0.001
Maximal weight of 2nd instar (g)	0.445	0.557	1,495.5	< 0.001
Maximal weight of 3rd instar (g)	4.468	6.571	541	< 0.001
Feeding period of 3rd instar (day)	35	40	1,206	< 0.001
RGR (g g^{-1} day^{-1})	0.0991	0.1024	3,400	0.236
Female				
Number of specimens	159	68		
Head capsule width of 2nd instar (mm)	2.7	2.9	1,959.5	< 0.001
Head capsule width of 3rd instar (mm)	5.5	5.8	2,068	< 0.001
Maximal weight of 2nd instar (g)	0.34	0.366	4,163.5	0.006
Maximal weight of 3rd instar (g)	2.916	3.2425	3,105	< 0.001
Feeding period of 3rd instar (day)	30	25	7,695	< 0.001
RGR (g g^{-1} day^{-1})	0.0917	0.0938	5,070	0.459

Adult Body Size

Body size (EL) of Chanthaburi population stag beetles was larger than the Bangkok population in both sexes and both types of specimens (wild-caught and breeding), but it was not significantly different in wild-caught females (Table V-2). Medians of the body size were shifted to larger size in breeding specimens of both populations and sexes, but significant differences of body size between wild-caught and breeding specimens were found only in males of Bangkok population (W = 1,748, *P* = 0.009) and females of Chanthaburi population (W = 1,030.5, *P* = 0.002).

Table V-2 Comparisons of adult body size of *A. chelifer chelifer* stag beetles between Bangkok and Chanthaburi populations

Properties	Bangkok	Chanthaburi	W	*P*
Male-wild specimens				
Number of specimens	60	80		
Median of EL (mm)	10.9	13.05	642	< 0.001
Minimum EL (mm)	8.2	8.8		
Maximum (mm)	12.8	15.3		
Male-breeding specimens				
Number of specimens	124	53		
Median of EL (mm)	11.5	13.2	444	< 0.001
Minimum EL (mm)	9.6	8.8		
Maximum (mm)	13	15.8		
Female-wild specimens				
Number of specimens	39	54		
Median of EL (mm)	10.9	11.15	871	0.157
Minimum EL (mm)	8.2	7.9		
Maximum (mm)	12.8	12.9		
Female-breeding specimens				
Number of specimens	135	58		
Median of EL (mm)	11.2	11.9	1,712	< 0.001
Minimum EL (mm)	8.3	10.4		
Maximum (mm)	12.9	13.2		

Body size of wild-caught specimens was highly overlapped between the two populations, but the overlap was distinctively smaller in breeding specimens (Figure V-4). According to Gini coefficient, body size variation of Chanthaburi population males was greater than females, and greater than Bangkok population. On the other hand, the variation was similar between males and females of Bangkok population (Figure V-5A). Comparisons between wild-caught and breeding specimens indicated that the size distribution was reduced in breeding specimens. Lorenz asymmetry coefficients of breeding Bangkok females and wild-caught Chanthaburi males were less than 1 indicating the inequality was due to a relative large number of small individuals (Figure V-5B).

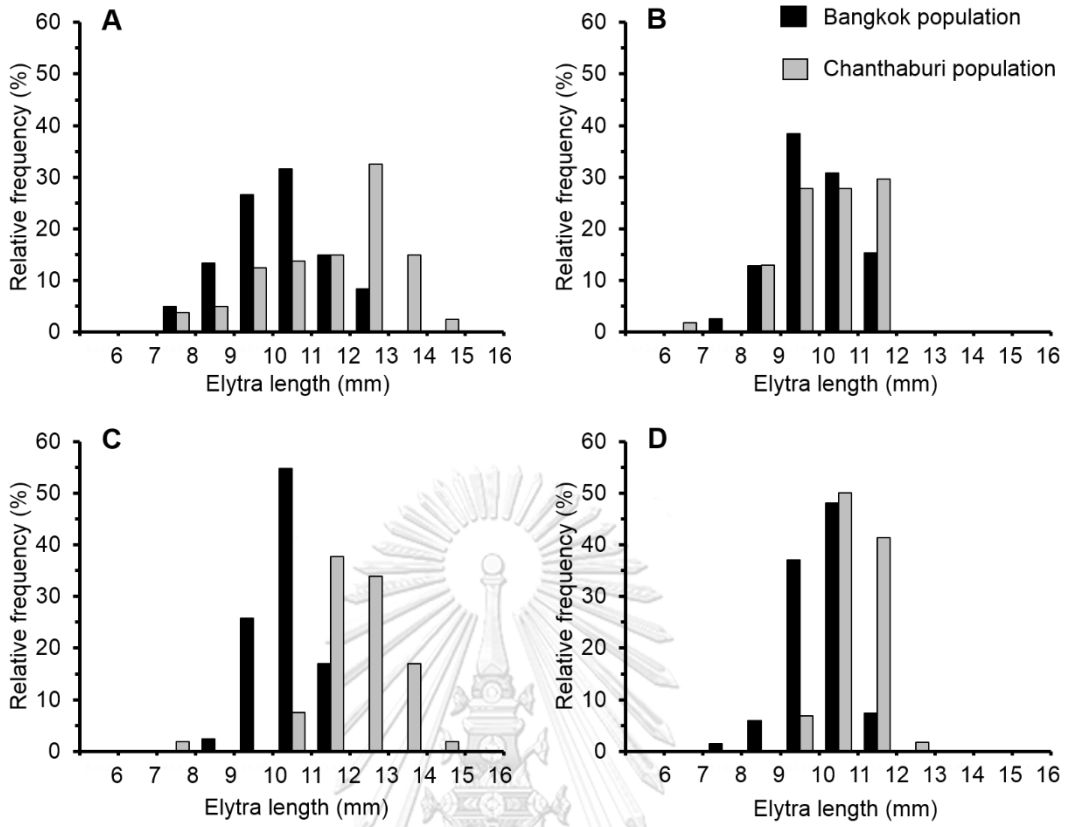

Figure V-4 Body size distribution of adult *A. chelifer chelifer* stag beetles, (A) wild-caught males, (B) wild-caught females, (C) breeding males and (D) breeding females.

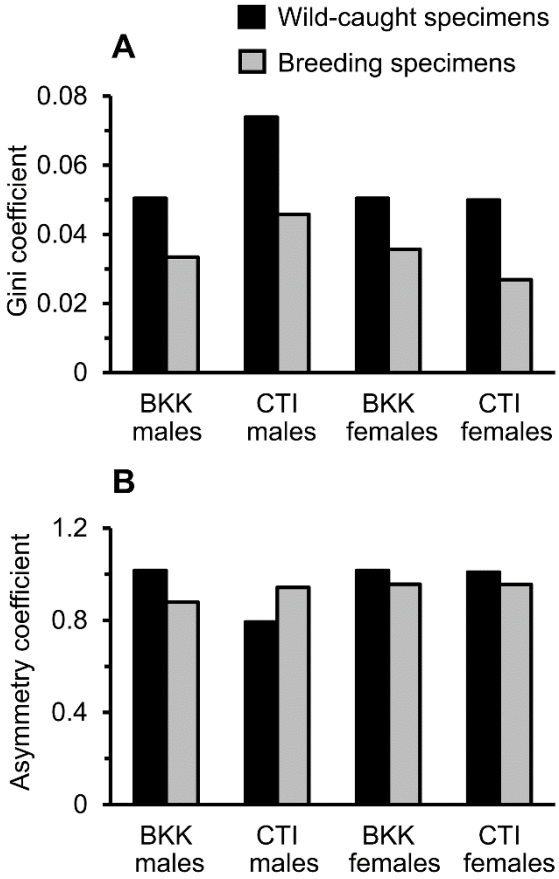

Figure V-5 (A) Body size inequality and (B) Lorenz asymmetry coefficients of adult *A. chelifer chelifer*. BKK and CTI refer to Bangkok and Chanthaburi populations, respectively.

Heritability

Regression analysis showed no significant relationship of body parts between parents and their offspring ($P > 0.05$) (Table V-3).

Table V-3 Parent-offspring regression between traits of adult *A. chelifer chelifer* stag beetles from Bangkok and Chanthaburi populations

Sex	Trait	h^2	F (df = 1, 8)	P
Bangkok				
Male	ML	-1.609	2.603	0.145
	EL	-0.211	0.190	0.674
Female	EL	0.879	1.865	0.209
Chanthaburi				
Male	ML	-0.094	0.224	0.650
	EL	-0.120	0.114	0.745
Female	EL	0.402	0.815	0.397

Allometry

Allometry between wild-caught and breeding specimens of the Chanthaburi population was significantly different with respect to both slope and intercept, while there was no difference in the Bangkok population (Tables V-4 and V-5). Comparisons between populations showed significant difference in the intercept of wild-caught specimens, and both slope and intercept of breeding specimens (Table V-6).

Table V-4 Allometric slopes (β_1) and intercepts (β_0) of mandible-elytra length relationship in major morph males of *A. chelifer chelifer*

Population	Parameter	Estimate	SE	t	*P*	MSE
Bangkok						
Wild-caught specimens (n = 45)						
	β_0	-1.897	0.347	-5.464	< 0.001	0.0063
	β_1	1.569	0.141	11.104	< 0.001	
Breeding specimens (n = 117)						
	β_0	-2.411	0.396	-6.087	< 0.001	0.0094
	β_1	1.785	0.162	10.989	< 0.001	
Chanthaburi						
Wild-caught specimens (n = 65)						
	β_0	-1.572	0.319	-4.935	< 0.001	0.0085
	β_1	1.459	0.124	11.790	< 0.001	
Breeding specimens (n = 52)						
	β_0	0.508	0.3100	1.640	0.107	0.0037
	β_1	0.690	0.120	5.744	< 0.001	

Table V-5 Comparisons of allometry between types of specimens (wild-caught and breeding) using analysis of covariance (ANCOVA)

Variation	df	Mean square	*F*	*P*
Bangkok population				
Slope				
$\log_e$(EL)	1	1.957	224.094	< 0.001
Population	1	0.007	0.806	0.371
Type × $\log_e$(EL)	1	0.008	0.921	0.339
Residual	158	0.009		
Intercept				
$\log_e$(EL)	1	1.957	224.205	< 0.001
Type	1	0.007	0.807	0.37
Residual	159	0.009		
Chanthaburi population				
Slope				
$\log_e$(EL)	1	1.286	194.94	< 0.001
Population	1	0.277	42.04	< 0.001
Type × $\log_e$(EL)	1	0.109	16.46	< 0.001
Residual	113	0.007		
Intercept				
$\log_e$(EL)	1	1.286	171.65	< 0.001
Type	1	0.277	37.02	< 0.001
Residual	114	0.007		

Table V-6 Comparison of allometry between stag beetle populations using analysis of covariance (ANCOVA)

Variation	df	Mean square	*F*	*P*
Wild-caught specimens				
Slope				
$\log_e$(EL)	1	3.331	423.410	< 0.001
Population	1	0.046	5.881	0.017
Population × $\log_e$(EL)	1	0.003	0.323	0.571
Residual	106	0.008		
Intercept				
$\log_e$(EL)	1	3.331	426.106	< 0.001
Population	1	0.046	5.919	0.017
Residual	107	0.008		
Breeding specimens				
Slope				
$\log_e$(EL)	1	5.020	641.33	< 0.001
Population	1	0.428	54.65	< 0.001
Population × $\log_e$(EL)	1	0.186	23.73	< 0.001
Residual	165	0.008		
Intercept				
$\log_e$(EL)	1	5.020	564.11	< 0.001
Population	1	0.428	48.07	< 0.001
Residual	166	0.009		

Discussion

Variation in body size of animals is typically affected by both genetic and environmental factors (Scheiner, 1993; Emlen and Allen, 2003; Lewis et al., 2012; Tsuchiya et al., 2012). However, body size due to environmental dependence is frequently reported in the beetle species that males are under high selection pressure

from male-male competition, such as rhinoceros beetle, dung beetle and stag beetle (Moczek, 1998; Shafiei et al., 2001; Moczek, 2002; Karino et al., 2004; Kawano, 2006; Whitman and Ananthakrishnan, 2009; Harvey et al., 2011a; Gotoh et al., 2014; Romiti et al., 2017). Normally, body size of these beetles are highly plastic in males (Tomkins et al., 2005; Kawano, 2006). Study in *L. cervus* indicated males had greater body size variation than females in all observed populations (Harvey and Gange, 2006; Harvey et al., 2011a). For this study, the high degree of body size variation in wild males of Chanthaburi population possibly imply the heterogeneity of environmental conditions in their habitats, while the lower variation and less size difference between sexes in Bangkok population may due to more habitat homogeneity.

The distinctive reduction of body size variation in the breeding stag beetles comparing to the wild beetles indicating the strong effects of environmental factors on the body size. Similar results have been reported in cactophilous fly *Drosophila buzzatii* Patterson & Wheeler, 1942, which the body variation of wild flies was greater than the flies reared under standard condition approximately four times due to temperature fluctuation and larval food supply in natural habitats (Robertson, 1987). Nevertheless, variation in body size was still observed in the breeding specimens that was possibly due to genotypic variance in the populations (Scheiner, 1993; Emlen and Allen, 2003).

From the breeding experiment, rearing temperature was in the range similar to natural habitats of both stag beetle populations and the diet for the larvae was sawdust of rubber trees, which is a non-native plant species of Thailand, thus, biased results from temperature and food preferences between populations could be diminished. By rearing under the same condition, the discrete of body size distribution and the

differences in static allometry between the two populations in breeding specimens probably implied differences in genetic basis relating to body size. Although, narrow-sense heritability (h^2) of the observed body parts was not detected within the populations, it should be noted that the sample size in this study was small that possibly resulted in lack of statistical power to clarify the heritability. Additionally, body size due to genetically based is mostly additive effects that sometimes requires observation more than one generation (Robertson, 1987; Emlen, 1996; Reeve et al., 2000). However, study in other stag beetles, such as *Cyclommatus metallifer* Boisduval, 1835, by breeding in captivity showed no narrow-sense heritability in absolute mandible length, but it was detected in static allometry between mandible length and body size (Gotoh et al., 2012).

The most important stage affecting the adult morphology is the last instar in which the larval growth is terminated and the imaginal discs have substantial proliferated to prepare the adult structures. (Emlen and Nijhout, 1999; D'Amico et al., 2001; Emlen and Nijhout, 2001; Davidowitz et al., 2003; Truman et al., 2006; Gotoh et al., 2011). This period is typically regulated by hormonal controls, and some were reported to associate with the acquired nutrients (Browder et al., 2001; Ikeya et al., 2002; Gotoh et al., 2014). Some physiological processes are heritable and gradually altered across several generations resulting in the change of average body size in a population, for examples critical weight and time to prothoracicotropic hormone (PTTH) secretion in *Manduca sexta* (L., 1763) (D'Amico et al., 2001; Davidowitz et al., 2003). This indicated the capability to develop the genotypic differences at larval stage between insect populations and also body size difference. From our results, only RGRs were similar between the populations, while developmental time was

significantly different. Developmental time of insects is often plastic, which can be affected by extrinsic factors (D'Amico et al., 2001; Shafiei et al., 2001; Goehring and Oberhauser, 2002). Study in dung beetles (Scarabaeidae) revealed that after larvae attained the critical weight, feeding of the larvae can be extended under high food quantity condition, but suddenly enter prepupal and pupal stages when the food is limited (Shafiei et al., 2001). At population level, it is possible that the selection may act on the mean critical weight, and consequently affect the difference of maximal weight and beyond to the adult body size. However, more extensive studies in stag beetles are needed for this matter.

Resource availability in a habitat has long been known as an essential component to force the body size alteration and microevolution in many organisms (Foster, 1963, 1964). Change in body size of a population is adaptive to improve fitness suitable for a habitat. Under highly resourceful environment, large body size gain advantages by enhancing competitive potential and reproductive success (Calvo and Molina, 2005; Karino et al., 2005; Kajita and Evans, 2010). On the other hand, small body size is more advantageous under resource limitation due to low food requirement which could reduce the risk from starvation (Blanckenhorn, 2000). The results of this study were consistent with the study in stag beetle *L. cervus*, that the individuals from urban areas had relatively smaller size when compared to populations from wood land (Harvey et al., 2011a). Stag beetles require decaying wood for larval growth. Trees and dead wood are limited resources in urban areas, therefore the body size selection would be shifted to the smaller size.

Considering the climate in Chanthaburi province, high rainfall and long rainy day in a year provide moisture in decaying wood, an important factor for growth of stag beetle larvae, for long period allowing more time to gain weight (Songvorawit et al., 2017a). In contrast, tropical savanna climate as in the Bangkok metropolitan areas has lower rainfall and shorter rainy days, that provides shorter time for supporting the growth. Another possible reason could be explained by habitat stability. Collecting sites of the Chanthaburi population was forest areas. After a tree dies in a forest, it will stay in that place and be eventually degraded that can last for several years to complete degradation (Schmidt and Czeschlik, 2006; Liu et al., 2013), and provide sufficient time for stag beetle larval development. On the other hand, dead trees in Bangkok metropolitan area, which is urban area, are often disturbed by human activities, such as burning, transferring to another place or made into woodchip and compost for aesthetic and safety reasons. Reduction of the developmental time may be an adaptive strategy of Bangkok population stag beetles to respond to such unpredictable condition and increase their fitness regarding on survival rate, and further results in the decrease in adult body size due to short time to gain weight.

However, it cannot be concluded that the directional selection of body size took place only with Bangkok population. Environmental conditions in the forest of Chanthaburi province also has potentiality to promote them to evolve in the direction to increase their body size by extending the development time (with sufficient food) in order to increase mating success of males and fecundity of females. Additionally, it needs more experiments to examine the differences between stag beetle populations could really improve their fitness to the local habitats, that will confirm these differences as an adaptive divergence.

CHAPTER VI

FEEDING PERFORMANCE RESPONSES TO FOOD AVAILABILITY OF STAG BEETLE *Aegus chelifer chelifer* (COLEOPTERA: LUCANIDAE) LARVAE

Abstract

Stag beetle larvae are saproxylic insects feeding on decaying wood which may be limited in quantity or availability in some habitats, such as urban park and tree plantation, due to human management. Stag beetles exploit these habitats may possess some traits to adapt under constraint condition. To evaluate the ability of stag beetle larvae to exploit food source and the effects of food quantity on their growth, feeding performances of tropical stag beetle *Aegus chelifer chelifer* MacLeay, 1819 (Coleoptera: Lucanidae) were investigated by rearing of larvae with different quantities of diet until adults. Apparent digestibility of the larvae was approximately 9% and conversion efficiency of the ingested food was ranging between 0.7%–1.7%. Feeding period, total food consumption and adult body size significantly increased with the increase of diet quantity. However, the total food consumption was still less than the amount of diet providing in the rearing containers even though the nutritional values of the remaining diet and faeces did not significantly decreased. Males had higher consumption rate than females due to shorter food retention time at the same weight. The differences of feeding performances depending on food availability may be an

adaptive plasticity of stag beetles to enhance their fitness under constraint condition and further result in body size variation of adults.

Keywords: conversion efficiency, digestibility, food retention time, growth rate, gut load

Introduction

Dead wood is a commonly available resource in many terrestrial ecosystems that is inhabited by various invertebrate fauna. However, lignocellulose, a major component of wood, normally resists to chemical and physical degradations and requires specific enzymes to degrade which are possessed by some microorganisms and arthropods. Thus, only some organisms are able to utilise wood as a food or energy source efficiently. (Kehler et al., 2004; Nordén et al., 2004; Schmidt and Czeschlik, 2006; Walczynska, 2007; Lachat et al., 2012).

Stag beetles are considered as saproxylic insects due to feeding on decaying wood during larval stage (Araya, 1993b, a; Wood et al., 1996; Meggs and Munks, 2003; Harvey et al., 2011a). Although the main food source of stag beetle larvae is decaying wood, food preference and microhabitats vary depending on the beetle species. For examples, many species, such as *Lucanus cervus* (Linnaeus, 1758), *Phalacrognathus muelleri* (MacLeay, 1885), *Prosopocoilus* spp. and *Dorcus* spp. were reported to have high density in decaying logs with the presence of wood-decaying fungi and some beetle species also had specificity to the decaying type of wood (Araya, 1993b, a; Wood et al., 1996; Ek-Amnuay, 2009; Tanahashi et al., 2009; Harvey et al., 2011a). Some stag beetles, such as *Odontolabis* spp. and *Lissotes* spp., habit as soil-dwellers feeding

on highly degraded wood underneath the decaying logs instead of living inside the logs (Meggs and Munks, 2003; Ek-Amnuay, 2009).

Distribution of most stag beetles is usually limited to forest where dead wood is abundant. However, there are some species found to live in habitats with limited resources, for example *Aegus chelifer chelifer* MacLeay, 1819 (Coleoptera: Lucanidae). This species is commonly and widely distributed in the mainland of Southeast Asia. They can be found in both forest and urban habitats and seem to be the only one stag beetle species lives in Bangkok metropolitan area, Thailand, where the amount of decaying logs is relatively low due to sanitation. (Mizunuma and Nagai, 1994; Pinratana and Maes, 2003; Ek-Amnuay, 2008). Moreover, *A. chelifer* was reported as an alien species in the Seychelles islands, Western Indian Ocean (Carpaneto et al., 2010). These indicated to their excellent survivability and adaptation to inhabit in various habitats.

Feeding performance is required to comprehend in abilities of wood exploitation and adaptation under limited resources of stag beetles. Moreover, many stag beetles have great variation in body size, especially males, within a population. It was believed that food availability was the major component regulating the growth of larvae and adult body size (Gotoh et al., 2011; Gotoh et al., 2012). Therefore, the experiment with *A. chelifer chelifer* stag beetles was conducted by breeding and rearing them in the laboratory to estimate the amount of food requirement and digestibility during larval stage. The effects of food quantity on their larval growth, feeding performances and adult body size were examined in order to clarify their adaptation to respond to habitats under limited food source.

Materials and Methods

Stag Beetles

A. chelifer chelifer stag beetles were initially collected from natural habitats, such as public parks and backyards, in Bangkok metropolitan area (Bangkok and Nonthaburi provinces; 13° 20' N to 14° 08' N and 100° 15' E to 100° 56' E), central plain of Thailand, during 2012–2014. They were bred and maintained in the laboratory for three to four generations prior to the experiments. Larvae were reared in 4,500 ml plastic containers (approximately 20–30 individuals per container) fully filled with fermented sawdust substrate as a diet (fermented sawdust of the rubber tree, *Hevea brasiliensis* Mull. Arg., supplemented with 10% (w/w) wheat flour, 60 ± 5% (w/w) water content, pH 7.5−8.0, modified from Ek-Amnuay (2009)) under constant darkness at 29 ± 4ºC and 75 ± 5% relative humidity. Female larvae can be identified from the presence of yellow ovoid shape of ovaries visible through the larval cuticle at the dorsal part of abdomen, while males lack this character (Fremlin and Hendriks, 2014).

Gut Load and Digestibility

Because stag beetle larvae live inside and feed on the sawdust substrate, the exact amount of ingested food could not be directly observed. Therefore, indirect method was conducted by using chromic oxide (Cr_2O_3) as a marker (Kimura and Miller, 1957; McGinnis and Kasting, 1964; Holter, 1973; Hendriksen, 1991; Köprücü and Özdemir, 2005). The diet was prepared by mixing the fermented sawdust with Cr_2O_3 in the ratio of 2 g Cr_2O_3 to 1 kg dry fermented sawdust and adjusted the moisture to 60% (w/w). Third instars with various weights, except for prepupal stage (identified from

weight decrease, turning of cuticle from clear to more turbid and beginning of cocoon construction), were randomly selected and individually reared in 200 ml plastic cups filled with 110 g (wet weight) of Cr_2O_3 diet under the same rearing condition as aforementioned. Faeces pellets presented in the rearing cups of the first two days were removed because they might be from the ingested food remaining in the guts before the experiment. Faeces pellets from Day 3 to Day 5 of the same individual were collected, pooled together and stored at -20°C until analysis of digestibility. The larvae at Day 7 were weighed and kept frozen at -20°C for estimation of gut load. To measure Cr_2O_3, the samples (larvae or faeces) were dried at 60°C until a constant weight. Then, the amount of Cr_2O_3 in the samples was determined using chlorine bleach method as described by Suzuki and Early (1991).

Food Retention Time

Rhodamine B dye was used as a marker in the diet for estimation of food retention time in larval guts. This dye has been used as a tracking marker and has low adverse effects on feeding behaviour and development of many insects (e.g. Blanco et al., 2006; Gayahan and Tschinkel, 2008; Mascari and Foil, 2009). The fermented sawdust was mixed with rhodamine B with the ratio of 2 g dye to 1 kg dry fermented sawdust and adjusted the moisture to 60% (w/w). Third instar larvae with various weights were randomly selected and allowed to feed the dye stained diet in 200 ml plastic cups containing 110 g of the wet diet for 3 h. Then, the larvae were removed, washed with distilled water and brought into 60 × 15 mm petri dishes fully filled with unstained diet. The presence of faeces was checked every hour. The faeces from ingestion of the dye stained diet had pink colour that was easily distinguished from the

brown colour of the unstained diet (Figure VI-1). Food retention time was estimated in the unit of hour from the time that allowed them to feed the dye stained diet to the time that pink faeces presented by using an assumption that stag beetle larvae started to feed the dye stained diet since the first hour.

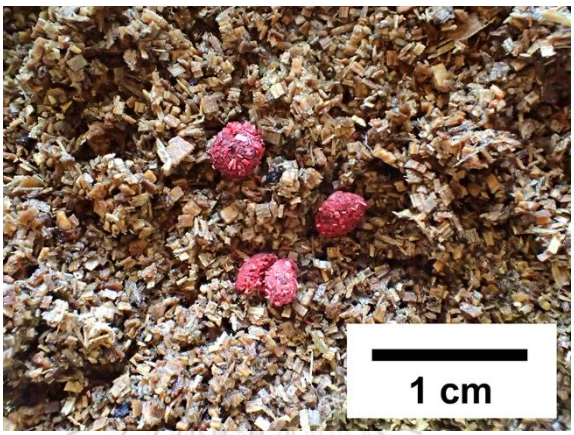

Figure VI-1 Pink faeces pellets of stag beetle larvae from ingestion of rhodamine B stained sawdust.

Effects of Food Quantity

Late second instars (head capsule width between 2.2–3.5 mm and weight > 0.2 g) were randomly selected and reared individually in plastic cups containing 28, 55, 110 and 220 g (wet weight) of fermented sawdust. Larval weight was measured to the nearest 0.001 g every five days until prepupal stage. After adults emerged, they were photographed using a digital camera (Olympus TG-4, Tokyo, Japan) and measured elytra and mandible length from the digital images to the nearest 0.1 mm using tpsDig2 software version 2.17 (Rohlf, 2013). The elytra length (used as proxy of body size) was measured along the middle line from the base of the scutellum to the posterior end of

the elytra, while the mandible length (used as proxy of weapon size) was measured as a straight line from the tip to the base of the left mandible.

Measurement of Nutritional Properties

Nutritional properties of faeces, diet before rearing and the remaining diet in the rearing cups after 30 days of rearing were measured. For collection of faeces, the experiment was conducted similar to the digestibility experiment, but stag beetle larvae were reared in normal diet (without marker). Faeces pellets from two larvae were pooled together (total larvae = 10). Samples were dried in a hot air oven at 60°C until a constant weight before measurements. Neutral detergent fibre (NDF) was analysed as described in Van Soest et al. (1991). Energy was analysed using bomb calorimeter (AC500, LECO Corporation). Thirty-day old diet in the rearing cup without larvae was used as a control. All samples were measured in five replicates.

Calculations of Relative Growth Rate, Gut Load, Food Retention Time and Consumption Rate

Relative growth rate (RGR) was calculated from the weight change in 15 days during the early stage of the third instars, which has exponential growth, shown in Eq. (6.1);

$$\text{RGR (g g}^{-1}\text{ day}^{-1}\text{)} = (\ln W_2 - \ln W_1) / t \qquad (6.1)$$

where W_1 is the weight at the first date of measurement at the third instars, W_2 is the weight after 15 days and t is the duration between measurement of W_1 and W_2 (15 days).

Gut load (GL) and apparent digestibility of the diet (AD) were calculated based on the formulae of Waldbauer (1968) as Eq. (6.2) and (6.3):

$$GL\ (g) = \text{Amount of } Cr_2O_3 \text{ in a larva / Amount of } Cr_2O_3 \text{ in 1 g wet diet} \quad (6.2)$$

$$AD\ (\%) = 100 - [100 \times (\%Cr_2O_3 \text{ in dry diet / } \%Cr_2O_3 \text{ in dry faeces}) \quad (6.3)$$

Relationships between larval fresh weight and gut load, and larval fresh weight and food retention time were firstly estimated from the scatter plots by visual inspection (Figure VI-3B, C). Since the relationships from the scatter plots were closed to linearity, the data were fitted to simple linear equations shown in Eq. (6.4) and (6.5) using ordinary least square (OLS) regression. Then, consumption rate in relation to larval weight was estimated from the gut load divided by the food retention time shown in Eq. (6.6). Total diet consumption during the third instar of each larva was estimated from the area under the curve by plotting the consumption rate at larva X g (CR_x) obtained from Eq. (6.6) against the feeding period of each stag beetle (data were obtained from the effects of food quantity experiment).

Gut load of larval weight X g (GL_x),

$$GL_x = \alpha_0 + \alpha_1 W_x, \quad (6.4)$$

Food retention time of larval weight X g (RT_x),

$$RT_x = \beta_0 + \beta_1 W_x, \quad (6.5)$$

Consumption rate of larval weight X g (CR_x),

$$CR_x = GL_x / RT_x \quad (6.6)$$

where W_x is the larval fresh weight, α_0 and β_0 are intercepts, and α_1 and β_1 are regression coefficients.

Conversion efficiency of the ingested food to the larval dry weight gain (ECI) was calculated from the growth during 15 days of the early stage of the third instars based on the formula of Waldbauer (1968) as shown in Eq. (6.7):

$$\text{ECI (\%)} = [(DW_2 - DW_1)/\text{Dry weight of food consumption}] \times 100 \quad (6.7)$$

where DW_1 and DW_2 are the dry weight of a larva at the first date of measurement and the dry weight after 15 days, respectively. The dry weight was estimated from the simple linear regression of the relationship between larval fresh and dry weight shown in Figure VI-3A, while the dry weight of food consumption is calculated by Eq. (6.6).

Statistical Analyses

Comparison of survivability between treatments was tested using Chi-square. Larval and adult performances from each treatment were compared using Kruskal-Wallis test, and Dunn's test was used for multiple comparison. Relationships between larval fresh weight and feeding performances were compared between sexes using analysis of covariance (ANCOVA). Statistical analyses were performed using the *base*, *FSA* and *dunn.test* packages in R version 3.3.0 (R Development Core Team, 2016).

Results

Effects of Food Quantity

Most stag beetle larvae (92.5%) could developed into adults (Table VI-1). Survivability was not significantly different between treatments (males: $\chi^2 = 0.03$, df =

3, P = 0.998; females: χ^2 = 0.01, df = 3, P = 1). Food quantity significantly affected larval growth and adult body size in both male and female. All measured traits, except for RGR of female larvae, were significantly different between treatments and sexes (feeding period: χ^2 = 46.6, df = 7, P < 0.001; RGR: χ^2 = 30.3, df = 7, P < 0.001; maximal larval weight: χ^2 = 74.1, df = 7, P < 0.001; elytra length: χ^2 = 73.6, df = 7, P < 0.001; male mandible length: χ^2 = 86.8, df = 7, P < 0.001; total diet consumption: χ^2 = 79.0, df = 7, P < 0.001), in which higher diet quantity yielded longer feeding period, higher RGR and maximal larval weight, larger adult body and weapon size, and higher diet consumption (Figure VI-2).

Table VI-1 Survivability of *A. chelifer chelifer* reared with different food quantity

Treatment	Sex	No. of larvae at the beginning	No. of larvae developed to prepupae[a]	No. of beetles died before adult stage	No. of beetles developed to adults[b]
28 g	Male	10	10	1	9 (90%)
	Female	16	10	1	15 (93.7%)
55 g	Male	14	13	1	13 (92.8%)
	Female	10	10	1	9 (90%)
110 g	Male	17	17	4	13 (76.5%)
	Female	11	11	-	11 (100%)
220 g	Male	19	19	-	19 (100%)
	Female	10	10	-	10 (100%)
Total	Male	60	59	6	54 (90%)
	Female	47	41	2	45 (95.7%)

[a] Number of samples used for analyses of larval performances.

[b] Number of samples used for analyses of adult performances. Percentage in parenthesis refers to survival rate from 3rd instar to adult.

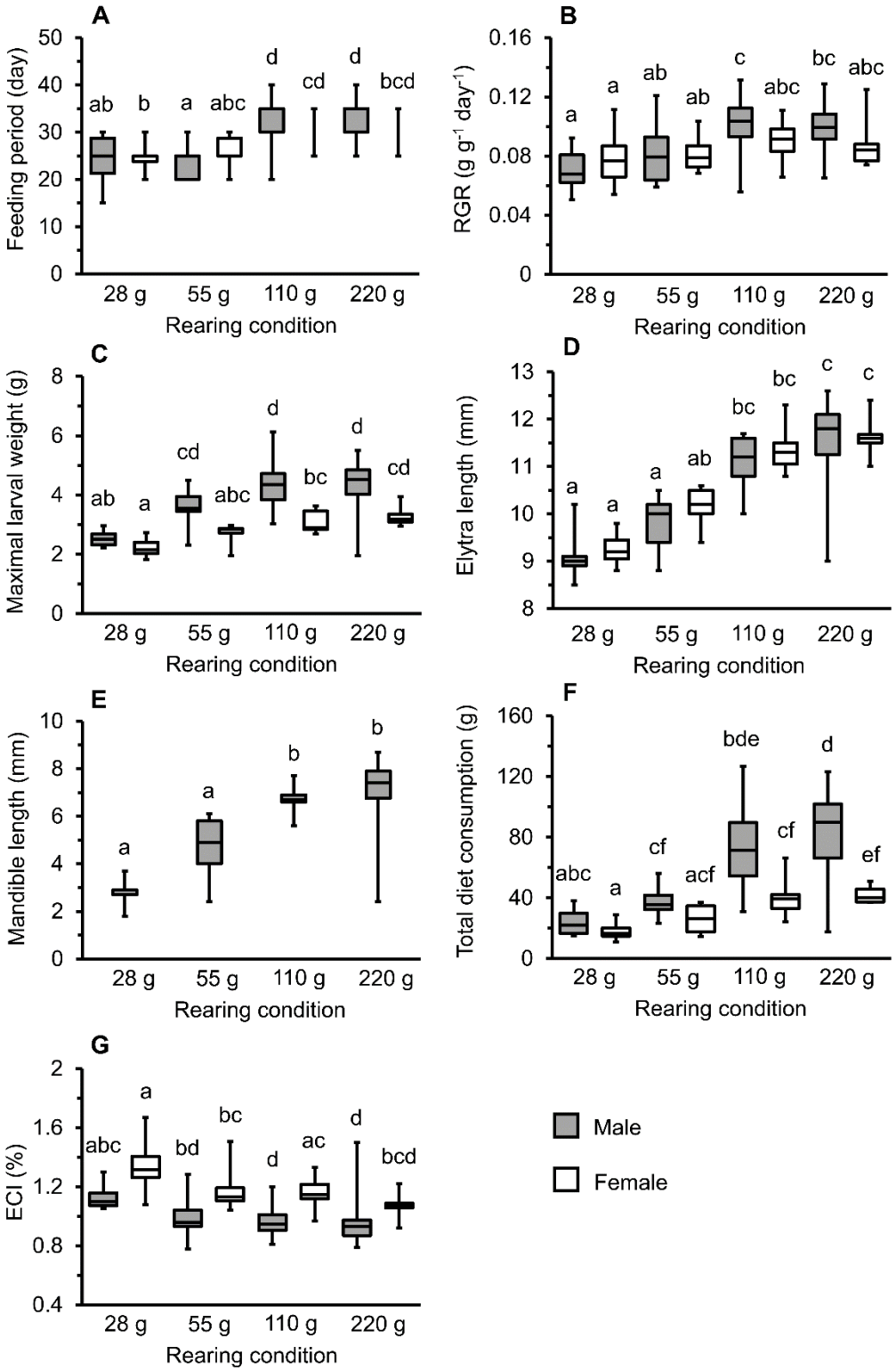

Figure VI-2 Effects of diet quantity on (A) feeding period of 3rd instars, (B) relative growth rate, (C) maximal larval weight, (D) elytra length, (E) male mandible length,

(F) total diet consumption of 3rd instars, and (G) conversion efficiency of the ingested food of *A. chelifer chelifer* stag beetles. Significant differences are denoted by different letters above the boxes (Dunn's test: $P < 0.05$).

Feeding Performances

Gut load or capability of stag beetle to hold the wet diet in their guts was approximately 25.2 ± 0.4% of larval fresh weight (n = 12, combined both males and females). The gut load was positively correlated to larval fresh weight (males: $r_s = 1$, df = 4, $P = 0.003$; females: $r_s = 0.94$, df = 4, $P = 0.017$), but was not significantly different between sexes (Figure VI-3B and Tables VI-2, VI-3). Food retention time varied ranging from 4 to 25 h and positively correlated to larval weight (males: $r_s = 0.91$, df = 14, $P < 0.001$; females: $r_s = 0.67$, df = 14, $P = 0.004$), indicated that food spent more time for passing through the guts in larger individuals. Comparison between sexes showed that the food retention time in the guts of female was longer than males at the same weight (Figure VI-3C and Tables VI-2, VI-3). Relationships between larval fresh weight and consumption rate were positive relation, which males had higher consumption rate than females at the same weight (Figure VI-3D).

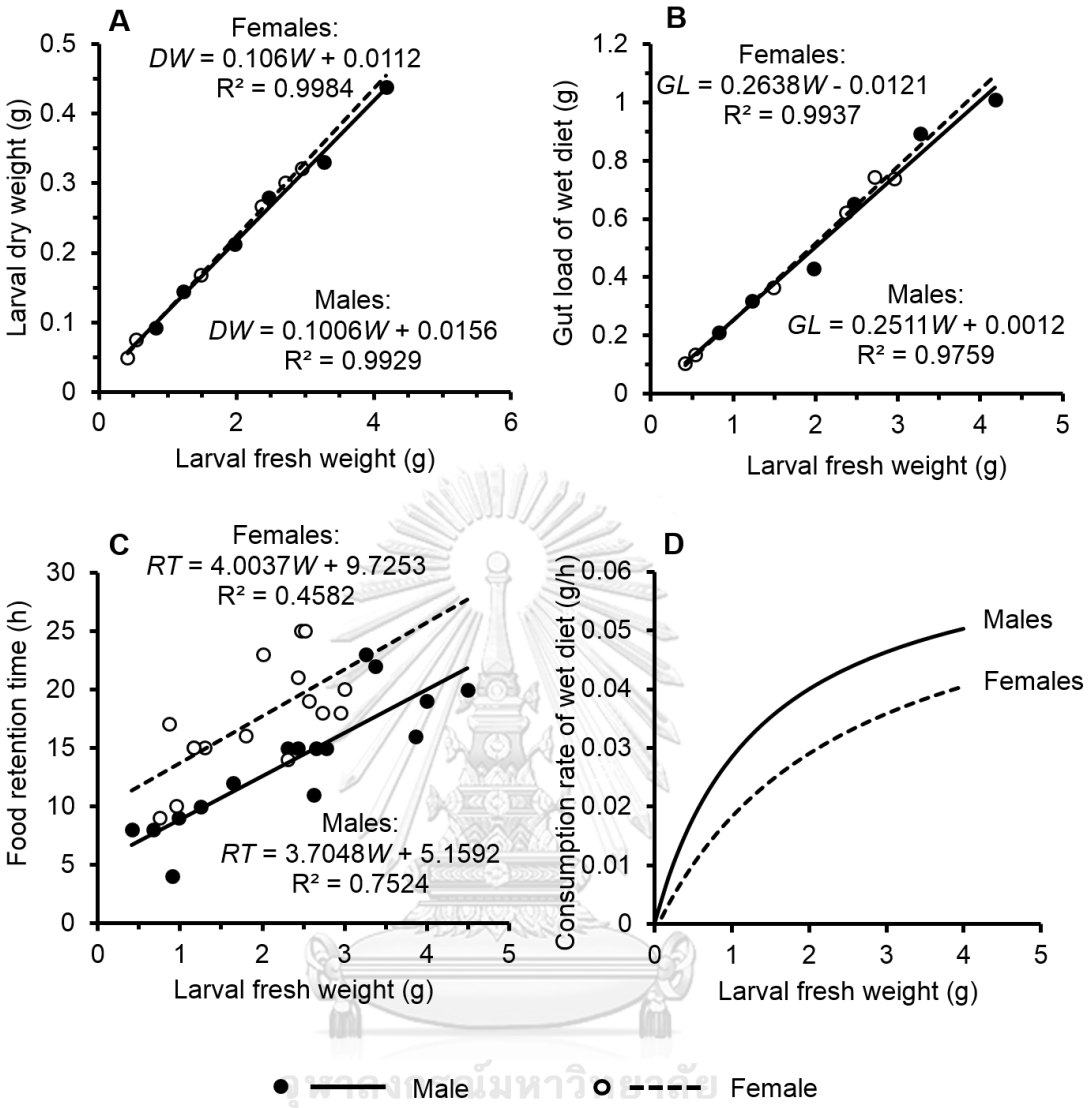

Figure VI-3 Relationships between larval fresh weight and (A) larval dry weight, (B) gut load of wet diet, (C) food retention time and (D) consumption rate of *A. chelifer chelifer* larvae. The equations represent the regression lines, where *W*, *DW*, *GL* and *RT* are larval fresh weight, larval dry weight, gut load and food retention time, respectively.

Table VI-2 Comparisons of the regression slopes of larval fresh weight and feeding performances relationships between males and females of *A. chelifer chelifer*

Variation	df	Mean square	*F*	*P*
Gut load				
Larval fresh weight	1	0.979	519.869	< 0.001
Sex	1	< 0.001	0.215	0.655
Larval weight × Sex	1	< 0.001	0.294	0.603
Residual	8	0.002		
Food retention time				
Larval fresh weight	1	378.4	37.108	< 0.001
Sex	1	206.4	20.241	< 0.001
Larval fresh weight × Sex	1	0.6	0.059	0.810
Residual	28	10.2		

Table VI-3 Comparisons of regression intercepts of larval fresh weight and feeding performances relationships between males and females of *A. chelifer chelifer*

Variation	df	Mean square	*F*	*P*
Gut load				
Larval fresh weight	1	0.979	564.142	< 0.001
Sex	1	< 0.001	0.234	0.64
Residuals	9	0.002		
Food retention time				
Larval fresh weight	1	378.4	38.35	< 0.001
Sex	1	206.4	20.92	< 0.001
Residuals	29	9.9		

Total Amount of Food Consumption

The total amount of food consumption during the third instar was estimated by assuming that their feeding performances were not affected by the amount of food quantity. The total amount of food consumption was significantly different between sexes and among treatments (χ^2 = 59.2, df = 7, $P < 0.05$) and increased with the food quantity (Figure VI-2F).

Digestibility, Conversion Efficiency and Nutrients

Apparent digestibility (AD) of stag beetle larvae was 9.74 ± 0.55% (n = 10, combined males and females together). Conversion efficiency (ECI) was significantly different among treatments of diet quantity and sexes, ranging between 0.7% and 1.7%, which females had higher ECI than males (Figure VI-2G). NDF of diets before and after rearing and faeces was similar. Energy of the diets before and after rearing was also similar, while energy of the faeces was significantly higher than the diets both at the beginning and after rearing (Figure VI-4).

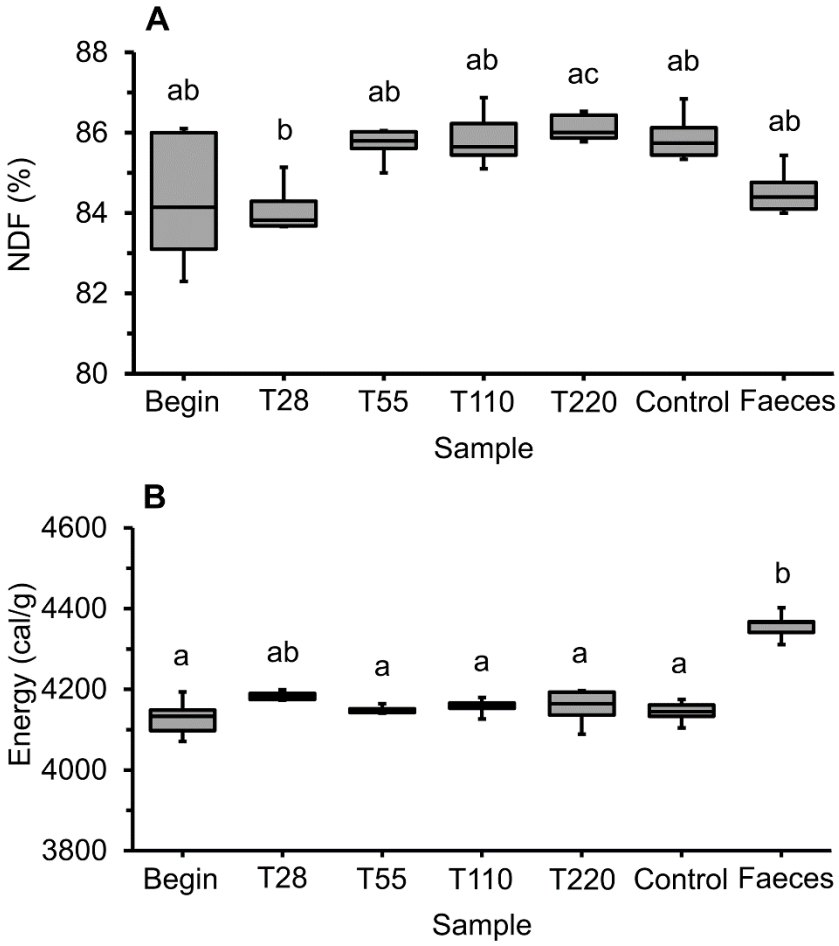

Figure VI-4 (A) NDF and (B) energy of faeces and diet from different treatments. Begin = diet before rearing (day 0), T28 = diet of treatment 28 g after rearing for 30 days, T55 = diet of treatment 55 g after rearing for 30 days, T110 = diet of treatment 110 g after rearing for 30 days, T220 = diet of treatment 220 g after rearing for 30 days. Control = 30-day old diet in rearing cups without larvae. Significant differences are denoted by different letters above the boxes (Dunn's test: $P < 0.05$).

Discussion

Body size variation is a dominant trait of many beetles in the superfamily Scarabaeoidea (Moczek, 1998; Kawano, 2002, 2003). Studies in dung beetle, rhinoceros beetle and stag beetle revealed that environment, especially nutrition, during larval stage, is the main factor that determined body size and weapon size (for males) in adults (Moczek, 1998; Shafiei et al., 2001; Moczek, 2002; Karino et al., 2004; Gotoh et al., 2011). Moreover, growth during larval stage and adult body size of these males tend to be more sensitive to environmental changes than females due to higher selective pressure from sexual selection (Tatsuta et al., 2004; Kawano, 2006; Emlen et al., 2007; Gotoh et al., 2011).

This study revealed that food quantity influenced feeding period, RGR and total amount of food consumption of *A. chelifer chelifer* larvae, and then resulted in adult body size difference among treatments. It is possible that other performances, such as food retention time in the guts and consumption rate, might be different among treatments and responsible for the differences in the growth and adult body size. Unfortunately, direct measurement could not be done due to lack of an appropriate procedure for study. It is well known that most stag beetles have sexual dimorphism in which males are usually larger than females (Mizunuma and Nagai, 1994; Kawano, 2003; Pinratana and Maes, 2003; Ek-Amnuay, 2008). Our results showed that the differences between sexes also included larval growth and feeding performances.

Amount of total diet consumption increased in relation to the increase of diet quantity. However, the amount of ingested food was still less than the exact amount of the diet in the rearing cups for all treatments even though nutrients (used NDF and

energy as proxies) in those diets after rearing were slightly changed from the beginning. This result was congruent with the estimation of food digestibility which was relatively low when compared to other xylophagous insects such as roaches and termites (Martin et al., 1991). The exact reason why energy in the faeces was higher than in the diets was unknown. Perhaps, the increase of energy (calorie) in the faeces may be from some physiological processes, microbial biomass inhabiting in the guts or the increase of lignin ratio in the faeces because it is difficult to be digested by insects and has relatively higher energy than other wood components (Kienzle et al., 2001; Dillon and Dillon, 2004; Engel and Moran, 2013).

Due to low food digestibility, this could explain why ECI of the stag beetles were relatively low when compared to other herbivorous insects, for examples 2–15% in longhorn beetles (Coleoptera: Cerambycidae) (Hosking and Hutcheson, 1979; Banno and Yamagami, 1989; Walczynska, 2007), 6–14% in nymphalid butterflies (Lepidoptera: Nymphalidae) (Banno, 1984), and 5–11% in the Colorado beetles (Coleoptera: Chrysomelidae) (Doležal et al., 2007). Theoretically, the larvae can consume the substrate (diet and faeces) for several cycles in the low diet quantity treatment (such as 28 g and 55 g treatments) due to the remaining nutrients in the rearing cups if disregards to the metabolic waste accumulation. To comprehend, physiology relating to digestive system of stag beetles is needed to be examined.

Wood is mainly composed of cellulose, hemicellulose and lignin, which required specific enzymes to degrade (Schmidt and Czeschlik, 2006). However, organisms that can produce effective enzymes to digest lignocellulosic components are restricted only in some groups (Schmidt and Czeschlik, 2006). There were evidences

that some insect species were able to produce their own enzymes for wood digestion, while most xylophagous insects need the assistance from microorganisms inhabiting inside the guts or colonizing in the food prior ingestion (Martin et al., 1991; Araya, 1993b, a; Cazemier et al., 1997; Hyodo et al., 2000; Dillon and Dillon, 2004; Geib et al., 2008). Currently, knowledge about capability of stag beetles to digest woody materials is sparsely reported. Observation in the fields indicated that habitats of stag beetle larvae were often associated with decaying wood infested by wood-decaying fungi (Araya, 1993b, a; Wood et al., 1996; Harvey et al., 2011b). In this case, woody polymers are partially degraded to be smaller molecules or are converted to be fungal biomass which are easier to be digested and assimilated by insects (Hanula, 1996). Experiment in larvae of *Dorcus rectus* (Motschulsky, 1857) by rearing them with mycelium of wood-decaying fungi could enhance larval performances and also supported that stag beetles obtain benefits from these microorganisms (Tanahashi et al., 2009; Tanahashi and Kubota, 2013).

Male stag beetles normally fight other males to protect their food sites and to access females. Larger males gain more chance to win the fights than smaller males, and thus gain more mating success (Okada and Hasegawa, 2005; Inoue and Hasegawa, 2013; Goyens et al., 2015b; Mills et al., 2016). From previous study in *A. chelifer chelifer* from the same population as this study, males could be divided into major (mandible length ≥ 5.1 mm) and minor (mandible length < 5.1 mm) morphs based on the allometry and mandible length (Songvorawit et al., 2017b). The treatment of 28 g diet gave only minor morph males, while the treatments 110 and 220 g gave nearly all major morph males. To be large adults, it requires sufficient food and time for development during larval stage. Perhaps, stag beetle larvae are able to estimate the

amount of their food via some mechanisms. Based on the experiment in dung beetle *Onthophagus taurus* (Schreber, 1759) (Coleoptera: Scarabaeidae), deprivation of the food during larval stage could accelerate the onset of metamorphosis into pupae and adults (Shafiei et al., 2001). Responses to low food availability by reducing the feeding duration and amount of food consumption may be an adaptive strategy to enhance their survivability under a constraint condition, while being under good condition or excess resource, they can optimise feeding performances to enhance fitness regarding to mating and reproductive success.

A. chelifer chelifer has been reported to be able to live under constraint condition as in urban and suburban areas where the quantity of decaying wood is relatively low (Ek-Amnuay, 2008; Songvorawit et al., 2017b). From our survey, the main places where these beetles lived in urban areas were public parks and backyards. Dead trees in these places are generally rare due to human management, such as for aesthetic and safety reasons, while small branches or woody debris are much higher in quantity. According to the results of this study, at least 28 g of diet was sufficient to support the growth of *A. chelifer chelifer* larvae. It was assumed that the first and second instars require very low diet quantity due to relatively smaller body size and shorter developmental time than the third instar. It means that only small pieces of decaying wood are sufficient for development to adults.

In conclusion, this study showed that the efficiency of woody substance utilization by *A. chelifer chelifer* larvae was relatively low. Nevertheless, the larvae could adjust their feeding performances depending on food availability that further resulted in adult body size variation. These were the evidence of plasticity in the growth

of stag beetle larvae, which might be an adaptive trait in order to improve their fitness under a constraint condition and could explain why *A. chelifer chelifer* stag beetle successfully inhabit in urban areas where the food source is limited. Low digestibility and ECI may be related to capability to digest woody materials. To more comprehension, study about digestive physiology is needed in the future.

CHAPTER VII

EFFECTS OF FOOD QUALITY, NITROGEN CONTENT AND WOOD-DECAYING FUNGI ON THE LARVAL PERFORMANCES AND ADULT BODY SIZE OF STAG BEETLE *Aegus chelifer chelifer* (COLEOPTERA: LUCANIDAE)

Abstract

Wood is typically difficult to digest and it also contains relatively low levels of some essential nutrients, especially nitrogen, for many insects. Stag beetle larvae are saproxylic insects feeding on decaying wood or wood infested with wood-decaying fungi, which is believed that it is a strategy to overcome the problems from wood utilization. However, the effects of food quality to stag beetles have not been proved, and microhabitats and food preference of stag beetle larvae can be varied depending on species. Therefore, the effects of food quality were investigated by rearing *Aegus chelifer chelifer* larvae with various sawdust-based diets. Fermentation of the sawdust resulted in significantly better larval growth performances and adult body size than the non-fermented sawdust. Nitrogen content gave positive effects to the growth of larvae, but 1.0% nitrogen content gave adverse effects probably due to the increase of pH in the diet. Rearing with all tested fungi gave negative effects to stag beetle larvae, which all larvae died before pupation. Furthermore, addition of supplements into the sawdust did not enhance the growth performances or adult body size.

Keywords: nutrition, sawdust, fermentation, larva, growth

Introduction

Wood contains a high proportion of carbohydrates which are mainly cellulose, hemicellulose and lignin (Schmidt and Czeschlik, 2006). These components are difficult to be ingested and digested by most animals due to its hardness and requirement of specific enzymes for digestion. Moreover, other essential components for growth of living organisms, such as nitrogen and some minerals, are very relatively low (Schmidt and Czeschlik, 2006).

Insects are dominant animals which are able to exploit woody materials efficiently. They have diverse strategies to overcome the problems of feeding on wood. Selection of nutrient-rich wood, such as high nitrogen content, for oviposition by female insects have been reported in some species to enhance growth performances of their offspring (Hosking and Hutcheson, 1979; Ayres et al., 2000; Saint-Germain et al., 2007; Saint-Germain et al., 2010). Another strategy of some insects is the association with fungi. Bark beetles and some eusocial insects, such as termites and leafcutter ants, are excellent examples of mutualistic relationships with the fungi (Ayres et al., 2000; Hyodo et al., 2000; Solomon et al., 2004). It is believed that fungi enhanced the growth of wood-feeding insects by altering lignocellulosic components to be easily digestible forms or suitable forms for assimilation (Hanula, 1996).

Stag beetles are classified as a saproxylic insect due to their feeding on decaying wood during larval stage. Empirical studies in natural habitats reported that they were frequently found in decaying wood infested by wood-decaying fungi and some species were also specific to the decaying type of wood (Araya, 1993b, a; Wood et al., 1996; Harvey et al., 2011a). Experiment by rearing stag beetle larvae of *Docus rectus*

(Motschulsky, 1857) with fungal mycelium as food revealed that they successfully grew under this condition which indicated that this species was fungivorous (Tanahashi et al., 2009; Tanahashi and Kubota, 2013).

Although most stag beetle larvae feed on decaying wood, their microhabitats are diverse and food preference can be different (Araya, 1993b, a; Wood et al., 1996; Meggs and Munks, 2003; Harvey et al., 2011a). *Aegus chelifer chelifer*, a small stag beetle species, is widely distributed in the mainland of Southeast Asia, both forest and urban habitats. In Thailand, they are reported as the only one stag beetle species found in Bangkok metropolitan area, where the amount of food (decaying wood) is limited (Pinratana and Maes, 2003; Ek-Amnuay, 2008). Due to ability to adapt in various habitats, it is interesting to know about the effects of food quality on their growth performances and adult body size. Previous study in the field indicated that preference on decaying logs of stag beetles, including *A. chelifer chelifer*, was related to decay class of logs or hardness of wood, nitrogen content and fungal biomass in those logs (Songvorawit et al., 2017a). Thus, it is possible that these factors may be the key components determining the growth and survivability of stag beetle larvae. To prove, the experiments were conducted by rearing *A. chelifer chelifer* larvae with various sawdust-based diets to examine their growth performances and adult body size under the given conditions.

Materials and Methods

Insects

A. chelifer chelifer stag beetles were originally collected from natural habitats in 2012–2014. Bangkok population stag beetles were collected from urban areas in Bangkok metropolitan area (Bangkok and Nonthaburi provinces; 13° 20' N to 14° 08' N and 100° 15' E to 100° 56' E), in the central plain of Thailand, while Chanthaburi population stag beetles were collected from forests in Chanthaburi province (12° 18' N to 13° 20' N and 101° 41' E to 102° 32' E), south-eastern Thailand. They were maintained in the laboratory for three to four generations before the experiments.

Breeding

Breeding was conducted by randomly paired stag beetles to mate. Mated females were released individually into 4.5 L breeding containers containing fermented sawdust of the rubber trees, *Hevea brasiliensis* Mull. Arg., supplemented with 10% (w/w) wheat flour and 60 ± 5% (w/w) moisture content (Ek-Amnuay, 2009).

Fungi

Four white-rot fungi, i.e. *Trametes lactinea* BCC33265, *Pleurotus ostreatus*, *Ganoderma lucidum* and *Lentinus polychrous*, and two brown-rot fungi, i.e. *Fomitopsis pinicola* BCC30879 and *Daldinia eschscholtzii*, were used in this study. Of these, *T. lactinea* BCC33265 and *F. pinicola* BCC30879 were purchased from BIOTEC Culture Collection Laboratory, Thailand, *P. ostreatus*, *G. lucidum* and *L. polychrous* were purchased from the Department of Agriculture, Thailand, and *D. eschscholtzii* was isolated from a decaying log in the dry-evergreen forest, Chanthaburi province,

Thailand. Fungi were maintained by culturing both in malt extract agar and in sorghum grains at room temperature. In addition, commercial sawdust spawns of *P. pulmonarius* for rearing stag beetle larvae purchased from two beetle shops, i.e. BeetleZ, Bangkok, Thailand and Siambeetle, Chiang Mai, Thailand, were also included for this study.

Diet Preparation

Sawdust of the rubber trees, *Hevea brasiliensis* Mull. Arg., was used as the main diet substrate for rearing stag beetle larvae. It was stored as dry sawdust in the dark at room temperature before use. Initial nitrogen content of the sawdust was 0.25%.

To study the effects of nitrogen content, the dry sawdust was pretreated to reduce the initial nitrogen level by soaking in water for five days, then the water was drained out. These steps were repeated again for three cycles. After that, the sawdust was divided into two portions. The first portion was fermented further for 15 days (with 60% moisture content in a container at room temperature). The second portion was mixed with casein enzyme hydrolysate with the ratio of 150 g casein to 1 kg dry sawdust and adjusted the moisture to 60%. The mixture was fermented in a container at room temperature for 15 days. After fermentation, the fermented sawdust was air-dried and stored in the dark at room temperature before use. Nitrogen contents of the fermented sawdust supplemented with casein and without casein were 1.01% and 0.05%, respectively. To make various nitrogen contents in the diet, the sawdust with casein was diluted with the sawdust without casein based on calculation.

To study the effects of fungi, sawdust spawns were made by mixing the dry sawdust (without any manipulation) with 10% (w/w) wheat flour and adjusted the

moisture to 60%. The mixture (220 g wet weight) was filled in 500 ml plastic cup and autoclaved at 121 °C for 1 h. For culturing of fungi, fungi from grain spawn were inoculated into the surface of the sawdust substrate in the cups approximately 20 grains per cup. The cups were incubated at room temperature. After the fungal mycelium fully grew in the cups (by visual inspection), they were incubated further for a week before use.

For the effects of supplements, three supplements, wheat flour, textured vegetable (TVP), and urea, were examined by mixing with the dry sawdust (without any manipulation) with 10%, 2% and 0.5% (w/w), and adjusted the moisture to 60%. The amount of supplements added in the sawdust was based on calculation to make each diet contained 0.50% nitrogen. Then, the mixtures were fermented for one month at room temperature. The diets were air-dried and stored in the dark at room temperature before use.

Rearing and Measurement

Stag beetles of Bangkok population were used as the main population for this study except for the experiment of effects of fungi that included both populations. Larvae at late second instar (head capsule width between 2.2–3.5 mm and body weight > 0.2 g) were randomly selected and reared individually in 500 ml plastic cups containing the diets (220 g wet weight, 60% moisture), under constant darkness at 29 ± 4 ºC and 75 ± 5% relative humidity. Head capsule width of the larvae was measured using a digital vernier caliper (Carbon Fiber Composites, Eagle One, Thailand) with the nearest 0.1 mm and larval weight using digital balance (OHAUS Adventure AR3130, New Jersey, USA) with the nearest 0.001 g every five days. Sex of stag beetle

larvae can be identified from the presence of yellow ovoid shape of ovaries in females which are visible through larval cuticle at the dorsal part of abdomen, while males lack this characteristic (Fremlin and Hendriks, 2014).

For measurement of adult morphology, they were photographed from dorsal view using a digital camera (Olympus TG-4, Tokyo, Japan). Each body part was separately measured from the digital images to the nearest 0.1 mm using tpsDig2 software version 2.17 (Rohlf, 2013). Mandible length (ML) was a straight line from the tip to the base of left mandible and elytra length (EL, used as proxy of adult body size) was the mid line from the base of the scutellum to the posterior end of the elytra).

Measurement of Diet Properties

Measurement of pH was conducted by mixing the diets with 2x (by weight) of distilled water, then the pH of the diet suspensions was measured by using pH meter (model pH 900, Precisa). Nitrogen content in the diets were analysed using a CHN analyser (LECO Corporation, 628 Series: CHN) as described in Vose and Swank (1993). Neutral detergent soluble (NDS) was analysed as described in Van Soest et al. (1991). Fungal biomass was measured as the glucosamine-equivalent as described in Ramachandran et al. (2005). All samples were measured in triplicate.

Statistical Analyses

Nutritional properties were compared using Wilcoxon rank-sum test and Kruskal-Wallis test. Survivability between treatments was compared using Chi-square. Larval and adult performances between treatments were compared using Kruskal-Wallis test, and Dunn's test was used for multiple comparison. Statistical analyses were

performed using the *base*, *FSA* and *dunn.test* packages in R version 3.3.0 (R Development Core Team, 2016).

Results

Effects of Fermentation of Sawdust

Nitrogen content, NDS, fungal biomass and pH of fermented and non-fermented sawdust were not significantly different (Table VII-1). Survivability of stag beetles was not significantly different between treatments (males: $\chi^2_1 = 0.01$, $P = 0.927$; females: $\chi^2_1 = 0.13$, $P = 0.722$) (Table VII-2). Fermented sawdust gave significantly higher maximal larval weight and RGR, while feeding period was similar (maximal larval weight: $\chi^2_3 = 39.11$, $P < 0.001$; feeding period: $\chi^2_3 = 5.45$, $P = 0.142$; RGR: $\chi^2_3 = 21.56$, $P < 0.001$) (Figure VII-1A, VII-1B and VII-1C). For adults, mandible length of males reared with fermented sawdust was significantly longer than males reared with non-fermented sawdust, while elytra length was similar between treatments and sexes (elytra length: $\chi^2_7 = 3.34$, $P = 0.342$; male mandible length: $\chi^2_3 = 7.11$, $P = 0.008$) (Figure VII-1D and VII-1E). External morphology of adults between treatments was also different. Cuticle of adults reared with fermented-sawdust was black as usual while cuticle of adults from non-fermented sawdust was brown as found in newly emerging adults indicating incomplete darkening of the cuticle (Figure VII-2A and VII-2B).

Table VII-1 Nutritional properties of sawdust-based diets and supplements

Diet	Nutritional properties (Mean ± SE)			
	N (%)	NDS (%)	Fungal biomass (mg/ml)	pH
Fermentation of sawdust				
Fermented	0.25 ± 0.00	10.6 ± 0.4	1.70 ± 0.01	7.5 ± 0.1
Non-fermented	0.27 ± 0.01	7.6 ± 0.5	0.54 ± 0.29	7.4 ± 0.1
	P = 0.076	*P* = 0.100	*P* = 0.100	*P* = 0.369
Nitrogen level				
0.05%	0.05 ± 0.00	9.6 ± 0.0	NA	7.5 ± 0.0
0.25%	0.25 ± 0.00	11.4 ± 0.1	NA	7.6 ± 0.1
0.50%	0.50 ± 0.01	14.1 ± 0.2	NA	8.0 ± 0.0
1.0%	1.00 ± 0.00	18.9 ± 0.7	NA	8.8 ± 0.1
	P = 0.013	*P* = 0.014	NA	*P* = 0.014
Wood-decaying fungi				
T. lactinea BCC33265	0.53 ± 0.02	21.2 ± 0.2	8.31 ± 0.42	4.9 ± 0.1
P. ostreatus	0.59 ± 0.00	18.4 ± 0.5	10.53 ± 0.48	5.4 ± 0.1
G. lucidum	0.51 ± 0.02	17.4 ± 0.8	5.01 ± 0.14	5.3 ± 0.1
L. polychrous	0.74 ± 0.04	19.7 ± 0.7	7.09 ± 0.50	5.0 ± 0.1
F. pinicola BCC30879	0.64 ± 0.00	30.4 ± 0.1	7.03 ± 0.48	5.2 ± 0.1
D. eschscholtzii	0.78 ± 0.04	15.3 ± 2.7	13.96 ± 1.47	6.3 ± 0.1
Commercial sawdust spawn from BeetleZ	0.61 ± 0.02	19.4 ± 0.2	12.75 ± 0.14	5.6 ± 0.0
Commercial sawdust spawn from Siambeetle	0.60 ± 0.01	19.6 ± 0.2	12.60 ± 0.26	5.5 ± 0.1
	P = 0.004	*P* = 0.017	*P* = 0.004	*P* = 0.004

[a] Nitrogen content was based on chemical formula.

[b] Faeces of three stag beetle larvae fed with sawdust supplemented with wheat flour.

NA = not available

Table VII-1 (Continued)

Diet	Nutritional properties (Mean ± SE)			
	N (%)	NDS (%)	Fungal biomass (mg/ml)	pH
Supplemented sawdust				
Wheat flour	0.44 ± 0.00	12.7 ± 1.4	NA	7.7 ± 0.1
TVP	0.48 ± 0.01	10.8 ± 0.4	NA	7.5 ± 0.1
Urea	0.54 ± 0.04	9.4 ± 0.3	NA	7.4 ± 0.1
	P = 0.238	P = 0.458	NA	P = 0.487
Casein hydrolysate	12.85 ± 0.00	NA	NA	7.2 ± 0
Wheat flour	1.63 ± 0.00	NA	NA	4.9 ± 0
TVP	9.0 ± 0.0	NA	NA	6.8 ± 0
Urea	46.7[a]	NA	NA	7.9 ± 0
Faeces[b]	0.69 ± 0.01	15.3 ± 0.4	NA	8.4 ± 0.1

Table VII-2 Survivability of *A. chelifer chelifer* reared with different diet quantity

Treatment	Sex	Number of samples		
		at the beginning	developed prepupal stage[a]	developed to adult stage[b]
Fermentation				
Fermented	Male	12	12	12 (100%)
	Female	17	17	17 (100%)
Non-fermented	Male	16	16	14 (87.5%)
	Female	18	17	10 (55.5%)
Nitrogen level				
0.05%	Male	8	7	1 (12.5%)
	Female	10	9	5 (50%)
0.25%	Male	8	6	1 (12.5%)
	Female	8	8	4 (50%)
0.50%	Male	7	6	2 (28.6%)
	Female	10	10	6 (60%)
1.0%	Male	9	8	2 (22.2%)
	Female	7	5	1 (14.3%)
Supplement				
Wheat flour	Male	7	7	6 (85.7%)
	Female	9	9	9 (100%)
TVP	Male	11	11	11 (100%)
	Female	9	9	8 (88.9%)
Urea	Male	8	8	8 (100%)
	Female	13	13	13 (100%)

[a] Number of samples used for analyses of larval performances.

[b] Number of samples used for analyses of adult performances. Percentage in parenthesis refers to survival rate from 3rd instar to adult.

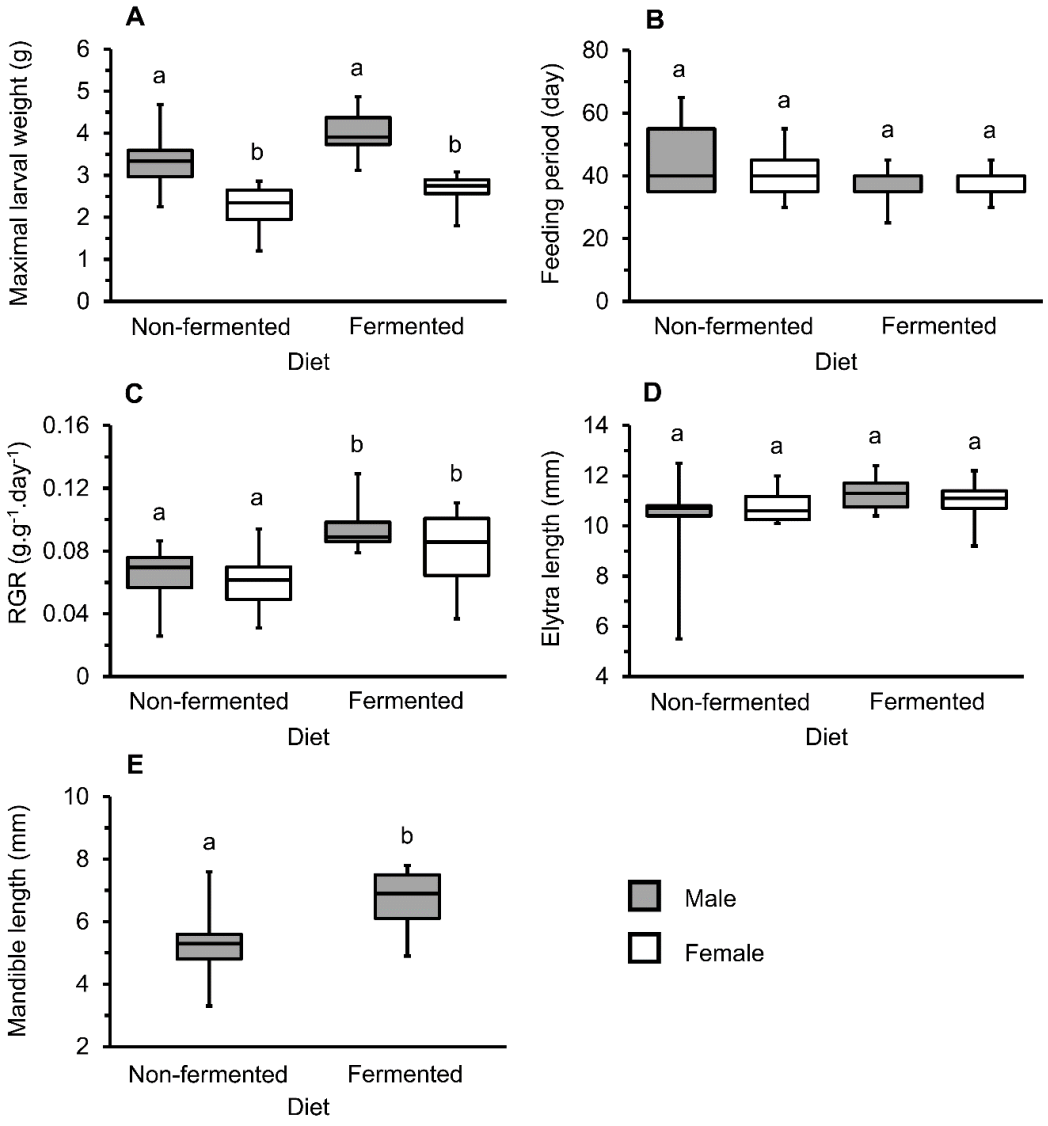

Figure VII-1 Effects of fermentation of sawdust diet on (A) maximal larval weight, (B) feeding period of 3rd instars, (C) relative growth rate of 3rd instar, (D) elytra length and (E) male mandible length of *A. chelifer chelifer* stag beetles. Significant differences are denoted by different letters above the boxes (Dunn's test: $P < 0.05$).

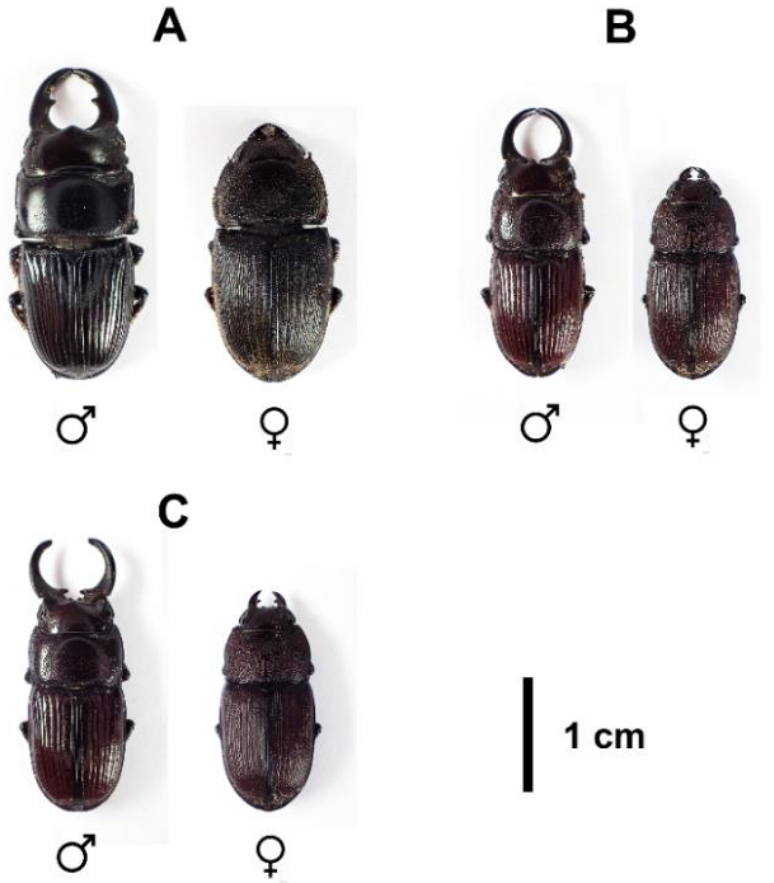

Figure VII-2 Cuticle colour difference of adult *A. chelifer chelifer* stag beetles after one month of emergence by rearing with (A) fermented sawdust, (B) non-fermented sawdust and (C) diet in the experiment of effects of nitrogen.

Effects of Nitrogen Level

NDS and pH were increased with the increase of nitrogen content in the diets (Table VII-1). Survivability of stag beetles was relatively low but was not significantly different between treatments (males: $\chi^2_3 = 0.10$, $P = 0.992$; females: $\chi^2_3 = 0.28$, $P = 0.964$) (Table VII-2). Maximal larval weight, feeding period and RGR were significantly different between treatments (maximal larval weight: $\chi^2_7 = 34.52$, $P < 0.001$; feeding period: $\chi^2_7 = 40.12$, $P < 0.001$; RGR: $\chi^2_7 = 33.77$, $P < 0.001$). Nitrogen

content at 0.50% gave the shortest feeding period and highest RGR in both males and females (Figure VII-3). Of these, maximal larval weight and feeding period of males were slightly higher and longer than females of the same treatments. Adult body size was not compared between treatments because the number of adult stag beetles was too low for statistical analysis. However, cuticle of adults from all treatments in this experiments was brown indicating incomplete darkening of the cuticle (Figure VII-2C).

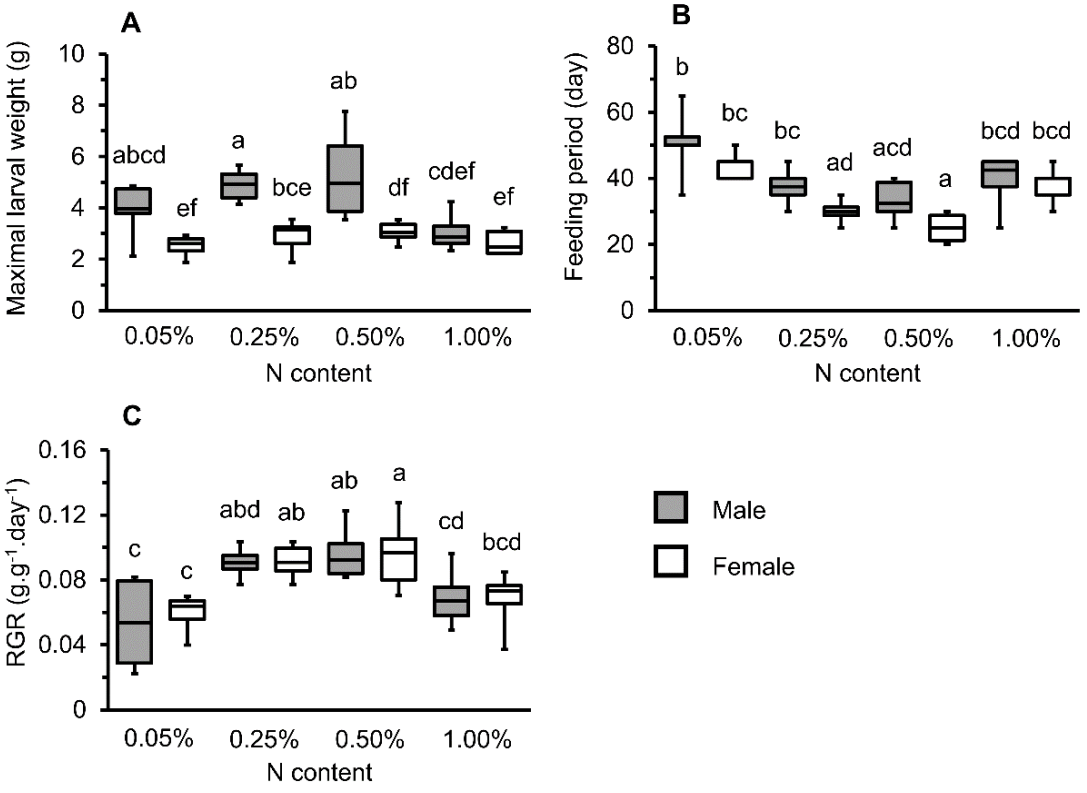

Figure VII-3 Effects of nitrogen content in diets on (A) maximal larval weight, (B) feeding period of 3rd instars and (C) relative growth rate of 3rd instar of *A. chelifer chelifer* stag beetles. Significant differences are denoted by different letters above the boxes (Dunn's test: $P < 0.05$).

Effects of Supplements

The observed nutritional properties of the diets with different supplements were similar (Table VII-1). Survivability of stag beetles was not significantly different between treatments (males: $\chi^2_3 = 0.01$, $P = 0.999$; females: $\chi^2_3 = 0.009$, $P = 0.1$) (Table VII-2). Larval performances were significantly different between some treatments (maximal larval weight: $\chi^2_7 = 62.41$, $P < 0.001$; feeding period: $\chi^2_7 = 35.71$, $P < 0.001$; RGR: $\chi^2_7 = 15.54$, $P = 0.030$), in which feeding period of larvae fed with wheat flour supplemented sawdust was slightly lower than the other groups (Figure VII-4A, VII-4B and VII-4C). However, comparisons of adult traits showed no significant differences among treatments (elytra length: $\chi^2_7 = 8.81$, $P = 0.267$ and male mandible length: $\chi^2_3 = 1.45$, $P = 0.694$) (Figure VII-4D and VII-4E).

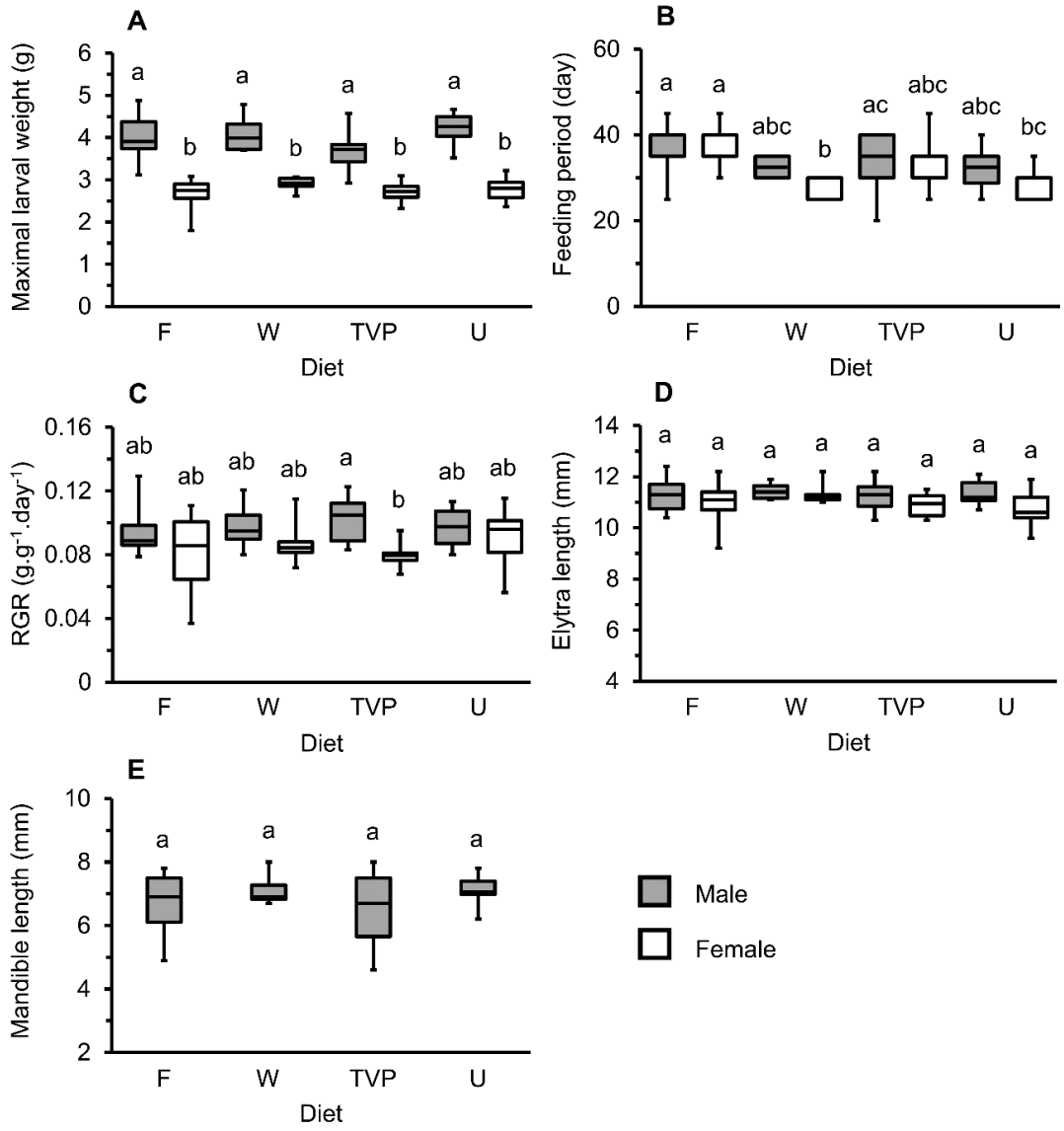

Figure VII-4 Effects of supplements in sawdust diets on (A) maximal larval weight, (B) feeding period of 3rd instars, (C) relative growth rate of 3rd instar, (D) elytra length and (E) male mandible length of *A. chelifer chelifer* stag beetles. Significant differences are denoted by different letters above the boxes (Dunn's test: $P < 0.05$).

Effects of Fungi

Although the substrate for making fungal spawn was the same as sawdust supplemented with wheat flour, the nitrogen content and NDS after culturing of fungi increased and the pH decreased into acidic condition (Table VII-1). Rearing of stag beetle larvae with sawdust spawns of all tested wood-decaying fungi and commercial sawdust spawns resulted in adverse effects to the larval growth. All second instar beetles could develop into third instars, but their weight gradually decreased and all beetles died within three weeks after the rearing (n = 10 for each tested fungi). Examination in the rearing cups showed that there were tunnels in the fungal substrate and faeces pellets (Figure VII-5A). Larval guts were full with the fungal substrate that they ate (Figure VII-5B). These indicated that the larvae were able to ingest the fungal substrate. Experiment by using larvae at early third instar (n = 5 for each tested fungi) and late second instars of Chanthaburi population (n = 5 for each tested fungi) were also conducted, but similar results were still obtained.

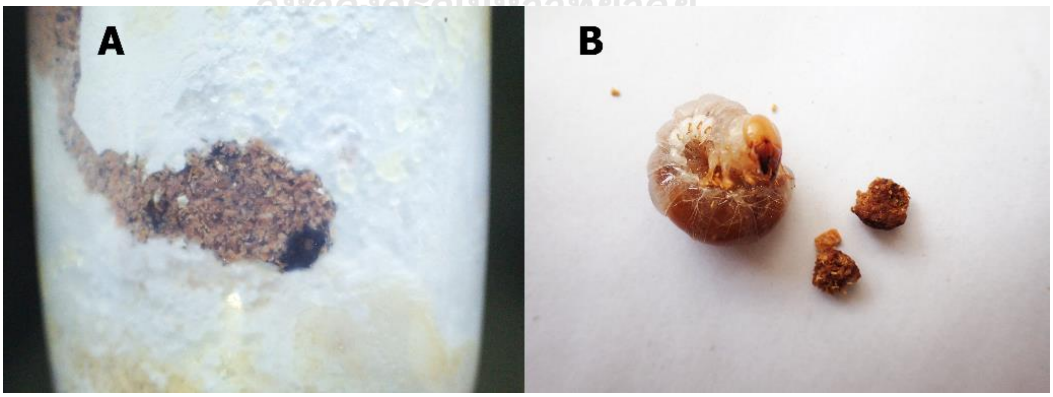

Figure VII-5 Rearing of *A. chelifer chelifer* larvae with sawdust spawn of wood-decaying fungi. (A) A tunnel in the sawdust spawn made by stag beetle larva and (B) stag beetle larva and its faeces after rearing in the sawdust spawn for five days.

Discussion

Stag beetle larvae are saproxylic insects feeding on decaying wood which physical and nutritional properties are typically different from intact wood (Araya, 1993b, a). From the results, RGR of the larvae fed with the fermented sawdust was higher than the non-fermented sawdust, while the feeding period was similar. Moreover, male stag beetles from fermented sawdust tended to invest resources during development into mandibles resulting in relative longer mandible length than those from non-fermented sawdust. This was similar to the study in *Gnatocerus cornutus* (F., 1798), that high diet quality did not affect body size, but resulted more investment in secondary sexual trait (Okada and Miyatake, 2010). This indicated that stag beetle larvae could consume or utilise the fermented sawdust more effectively than the non-fermented sawdust. Although sawdust is small wood particles, but the grains are still hard. Fermentation of sawdust in this experiment is to mimic the decaying process of wood in nature (Ek-Amnuay, 2009). Normally, decaying wood is softer and easy to be ingested by insects than intact wood. Moreover, long chains of lignocellulosic fibres are also partially degraded to be shorter molecules which facilitates digestion and utilization by wood-feeding insects (Araya, 1993a; Hanula, 1996; Schmidt and Czeschlik, 2006).

Previous study in the field indicated that nitrogen and fungal biomass contents in logs were the major factors relating to oviposition preference of female stag beetles including *A. chelifer chelifer* (Songvorawit et al., 2017a). This study revealed that nitrogen content affect larval performances by shortening feeding period and enhancing RGR. However, high nitrogen content in the sawdust also gave negative effects to larval

growth as seen in the treatment of 1.00% nitrogen. Diet pH increased with the increase of nitrogen content. Ammonia odour was also smelled from the diet with 1.00% N. Normally, ammonification and nitrification are parts of the nitrogen cycle that change nitrogen in organic matters into ammonia, nitrite and nitrate by activities of bacteria inhabiting in the substrate (Pajares and Bohannan, 2016). Ammonium ion is alkaline by itself that can increase pH of the surrounding environment, while high levels of nitrite and nitrate have been reported being toxic to animals (Wolff and Wasserman, 1972; Bruning-Fann and Kaneene, 1993; Cockburn et al., 2013). Although the amount of these compounds in the diets was not measured, it was possible that they would be responsible for the decrease of larval growth in the diet with high nitrogen content. However, it should be noted that decaying wood in nature can have nitrogen content over 1% of dry weight without the increase of pH into alkaline condition because nitrogen is normally incorporated within plant cell wall or microbial cells colonizing in the decaying wood (Saint-Germain et al., 2007; Tanahashi et al., 2009; Songvorawit et al., 2017a). This differs from the diets in this experiment which the nitrogen sources were outside the wood particles and could be transformed by microbial activities during the fermentation process.

Mortality of the larvae in the experiment of nitrogen effects was relatively high when compared to the other experiments. The death of the larvae was probably caused by the loss of some essential nutrients during the procedure of diet preparation. Soaking and washing the sawdust may not only reduce nitrogen, but also remove other nutrients, probably water-soluble minerals, from the sawdust. The lack of some nutrients would be responsible for incomplete sclerotization (cuticle hardening and darkening) of beetle cuticle resulting in brown cuticle (Schofield et al., 2003; Cribb et al., 2010).

Besides carbohydrates, other essential nutrients for animal growth in wood are very relatively low (Schmidt and Czeschlik, 2006). However, decaying wood may have more nutrients than living or intact wood due to decomposition process and activities of microorganisms and some arthropods inhabiting in those decaying wood. For example, wood-decaying fungi can increase nitrogen and other minerals in fallen logs by uptaking these nutrients from surrounding environment through their mycelia (Hanula, 1996; Tanahashi et al., 2009). According to rearing stag beetle larvae for commercial purpose, many recipes for diet preparation prefer to add supplements, such as wheat flour and rice bran, into the woody substrate in order to mimic decaying wood in nature and to enhance the growth of beetle larvae (Ek-Amnuay, 2009). Before the experiments, it was hypothesised that the addition of supplements into the sawdust would increase the growth performances of stag beetles due to the increase of nutrients, such as nitrogen and other essential minerals. However, the results showed no difference of larval performances and adult body size. It is possible that the initial nutrients in the sawdust is at the level that results in optimal growth for the larvae. Another explanation is probably related to the gut microorganisms, which has been reported inhabit in the guts of many wood-feeding insects, including stag beetles (Martin et al., 1991; Cazemier et al., 1997; Tanahashi et al., 2010; Fischer et al., 2013). *A. chelifer chelifer* stag beetle larvae may obtain more nutrients from the assistance of these symbionts instead of using wood-decaying fungi as other stag beetles (Araya, 1993b; Tanahashi et al., 2009; Harvey et al., 2011a; Tanahashi and Kubota, 2013). Analysis of their faeces showed that the nitrogen content was higher than the diet before ingestion. Therefore, addition of supplements probably no longer increase the growth of stag beetle larvae.

Stag beetles in natural habitats are often reported to have high occurrence in decaying logs infested by wood-decaying fungi and some species were also specific to the type of fungi in logs (Araya, 1993b, a; Wood et al., 1996; Harvey et al., 2011a; Harvey et al., 2011b). Experimentally, the growth of *D. rectus* stag beetle larvae could be enhanced by rearing with fungal mycelium as single diet (Tanahashi et al., 2009; Tanahashi and Kubota, 2013). Extracellular enzymes produced from wood-decaying fungi are highly effective to degrade lignocellulosic components of wood and convert to smaller molecules and then become fungal biomass which is easy to be digested and utilised (Hanula, 1996; Schmidt and Czeschlik, 2006). Surprisingly, this study revealed that wood-decaying fungi gave negative effects to the growth of *A. cheilifer chelifer* larvae. This result contradicted to the expectation before the experiment. Although *A. chelifer chelifer* from Chanthaburi population, which has been reported to be frequently found in logs containing relatively high fungal biomass, was included in the experiment, the same result as in Bangkok population stag beetles was still obtained (Songvorawit et al., 2017a). Since stag beetles could ingest the fungal diet, the problems were probably from the digestion and utilisation. Perhaps, this species may have alternative mode to obtain nutrient from wood instead of the use of helping from wood-decaying fungi as found in most stag beetles. Therefore, study in the digestive physiology of this species is necessary.

CHAPTER VIII

RESOURCE HOLDING POTENTIAL AND THE OUTCOME OF AGGRESSIVE INTERACTIONS BETWEEN *Aegus chelifer chelifer* (COLEOPTERA: LUCANIDAE) MALE STAG BEETLES

Abstract

Relationships between male stag beetles are usually aggressive interactions by using their long mandibles as weapons to compete with rival males over females. Males have great variation of body size in a population, thus their size should affect their behaviours and the outcome of fights. However, aggressive responses in relatively large body size and long mandibles stag beetles may differ from those small species, therefore aggressive interactions between male *Aegus chelifer chelifer* MacLeay, 1819, a small tropical stag beetle species, were investigated. Morphological traits in relation to the outcome of fight were tested. Multiple logistic regression revealed that the combination of body parts, i.e. pronotum length, pronotum width and elytra length was the best predictor for the outcome, while weapon size was less important. By using pronotum width as a resource holding potential (RHP), male with greater RHP had higher probability to win the combat when compared to males with relatively smaller RHP. There were no significant relationships between the size of morphological traits, including morph type, and initiation of the fight or aggressive intensity. Relationships between fight duration and RHP was not significantly consistent with any assessment strategies, but it was close to the mutual assessment.

Keywords: fight, dimorphism, assessment, mandible, body size, variation

Introduction

Interactions among conspecific members, regardless relationships within a family, are usually in the form of competition to be an ownership over limited resources, such as food, territories and mates (Siva-Jothy, 1987; Bridge et al., 2000; Hoem et al., 2007; Briffa and Elwood, 2009; Inoue and Hasegawa, 2013). In some situations, encounters between two or more individuals are unavoidable resulting to agonistic interactions between them. However, these interactions are costly which those animals have to pay energy, time or the risk of injury as a tradeoff (Siva-Jothy, 1987; Briffa, 2008; Arnott and Elwood, 2009; Briffa and Elwood, 2009).

Agonistic interactions of animals can be exhibited in various forms, such as displaying a certain performance to tell their strength and quality, engaging in direct conflict, or performing a series of behaviours from the lowest to highest aggressive intensity, that depend on species, resource value, resource holding potential (RHP), experience and mechanism to obtain information during the interactions (Siva-Jothy, 1987; Payne and Pagel, 1997; Hofmann and Schildberger, 2001; Pratt et al., 2003; Jennings et al., 2004; Goyens et al., 2015b). Under symmetric resource value, individuals with larger body size normally win the combat, but other traits, especially secondary sexual traits, can also influence the outcome and may be used as a predictor if those traits are reliable to indicate fighting potential, for example eyespan of stalk-eyed flies, horn length of Japanese horned beetles and chelae of shore crabs (Sneddon et al., 1997; Karino et al., 2005; Small et al., 2009).

Normally, fight between two individuals is terminated by the individual which gives up from the fight sooner, or known as “loser”. From the behavioural game theory perspective (Smith, 1982), relations of fight duration and RHP of the contestants are of interest topics about how decisions to escalate or give up from the fights is made (Briffa, 2014). According to Arnott and Elwood (2009), assessment strategies of fighting ability could be classified into three main mechanisms, based on the gathering information methods by contestants as follows. (1) Pure self-assessment, which each contestant knows only its own abilities and the decision to give up is made when the cost of fight of one contestant (loser) exceeds a threshold. (2) Cumulative assessment, which is similar to the pure self-assessment, but the actions of the opponents can inflict on the contestants resulting in acceleration of the cost reaching to the threshold point. Individuals which have higher threshold and/or better ability to inflict the cost of the opponents will be winners. (3) Mutual assessment, which each contestant assesses opponent’s RHP relative to their own. Thus it gives advantages to both contestants by reducing the cost of fight when an asymmetric combat takes place.

Body and weapon sizes do not only determine the fighting success, but also influence their fighting behaviour (Siva-Jothy, 1987; Emlen, 1997a; Karino et al., 2005; Cook and Bean, 2006). Small males of rhinoceros and horned dung beetles usually avoid direct conflict with other males and use alternative strategy, such as sneaking tactic, to access females without fight (Siva-Jothy, 1987; Emlen, 1997a; Karino et al., 2005; Kijimoto et al., 2013). For stag beetles, males are equipped with large mandibles using as a weapon for male-male combat in order to access females or protect territories mostly at sap exudates on tree trunks (Hongo and Okamoto, 2013; Hongo, 2014). Size and mandible shape of male stag beetles are highly variable across species and within

a population (Mizunuma and Nagai, 1994; Shiokawa and Iwahashi, 2000a; Pinratana and Maes, 2003; Ek-Amnuay, 2008; Inoue and Hasegawa, 2013). Thus it is possible that behaviours from male-male interactions vary according to morphological variation. However, most studies of fighting behaviour limited to the species possessing relatively large body size and long mandibles, such as *Prosopocoilus dissimilis okinawanus* Nomura, 1962 (Shiokawa and Iwahashi, 2000b), *P. inclinatus* (Motschulsky, 1857) (Hongo and Okamoto, 2013; Inoue and Hasegawa, 2013), *Lucanus maculifemoratus* Motschulsky, 1861 (Hongo and Okamoto, 2013) and *Cyclommatus metallifer* Boisduval, 1835 (Goyens et al., 2015b; Goyens et al., 2015a)

Therefore, fighting behaviour was examined in male stag beetles of *Aegus chelifer chelifer* MacLeay, 1819, a small stag beetle that is widely distributed in the mainland of Southeast Asia (Mizunuma and Nagai, 1994; Pinratana and Maes, 2003; Ek-Amnuay, 2008). Males are equipped with long, curved mandibles, while females have a smaller body size and shorter mandibles. From previous study (Songvorawit et al., 2017b), males of this species show dimorphism in morphology based on the mandible length. The relations of size and their aggressive behaviours and fighting success were examined. Assessment strategy that stag beetles used during the contests was also examined by comparing to theoretical models in order to understand more of the agonistic interactions of stag beetles.

Materials and Methods

Insects

Parental generation of *A. chelifer chelifer* stag beetles were collected from natural habitats in Bangkok metropolitan area (13° 20′ N to 14° 08′ N and 100° 15' E to 100° 56' E), central Thailand. These stag beetles were bred and the larvae were individually reared with fermented sawdust substrate supplemented with wheat flour modified from Ek-Amnuay (2008) under constant darkness at 29 ± 4 ºC. After they became adults, each beetle was individually maintained in a plastic cup containing moist tissue paper at 29 ± 4 ºC under 12:12 dark/light cycle and *ad libitum* fed with ripen banana. Only healthy (no observed injury) and virgin males with adult age between four and eight months were used in the experiments.

Morphological Measurement

Measurement of body parts was conducted by photographing stag beetles from the dorsal view with a digital camera (Olympus TG-4, Tokyo, Japan), then the mandible length (ML), head length (HL), pronotum length (PL), elytra length (EL), head width (HW) and pronotum width (PW) were measured from the digital images to the nearest 0.1 mm using the tpsDig2 software version 2.17 (Rohlf, 2013). Males were classified into two morphs based on previous study in *A. chelifer chelifer* (Songvorawit et al., 2017b), which minor morph males had mandible length < 5.1 mm and major morph males had mandible length ≥ 5.1 mm. In this study, total of 134 males were used in the experiment, 19 and 115 individuals were classified as minor morph and major morph, respectively.

Behavioural Experiment

The experiment was conducted between 21:00 to 03:00 hours under dim red light condition (110 lux). The arena for the contest was made from round plastic container with the size of 15 cm in diameter and laid with EVA foam sheet at the bottom. Males were randomly matched before the contest. Two males were introduced and acclimatised in a small glass cup lied at the opposite side for 15 min before experiment. Then, the males were allowed to meet each other and recorded their behaviour using a digital camera (29 fps, Olympus TG-4, Tokyo, Japan). A loser was defined from the individual that retreated from the combat, or feigned death, or flipped over and could not get back to the fight again within 30 sec, while another male was considered as a winner. Fight duration was counted when both males began engaging in the fight until the losers could be defined. The arenas were cleaned with 95% ethanol every time before the next trials. Each male was tested three times, unless they died or had injury, with one week interval in order to minimise an effect of fighting experience. The same pairs were not used as opponents on later trials.

Data Analyses

Effects of morphological traits on aggressive intensity were analysed using Kruskal-Wallis test and Dunn's test was used for multiple comparison. Ratio of males engaged in fights was compared between male morphs using Chi-square test. To find the best predictor for the outcome of aggressive interaction, Wilcoxon signed-rank test and multiple logistic regression with stepwise backward elimination procedure were used by using all morphological traits as independent variables and the outcome of the interaction as a dependent variable (win or lose). The best predictor was deemed as the

RHP of fight for *A. chelifer chelifer* males. Relationship between relative difference of RHP and outcome of fight was tested with logistic regression. Morphological traits affecting the initiation of aggressive behaviour was tested by Wilcoxon signed-rank test. The effect of initiation of aggressive behaviour on the outcome of aggressive interactions was analysed using Chi-square test. To discriminate whether the contests were resolved by which assessments, correlation test between fight duration and the RHP was conducted using Spearman's coefficient of rank correlation. assessment strategy was identified by following the suggestions by Taylor and Elwood (2003) and Arnott and Elwood (2009) as shown in the Table VIII-1. Testing pairs without fight (aggressive intensity < level 2, see the results) were excluded from these analyses. Statistical analyses were conducted using *base*, *FSA* and *dunn.test* packages in R version 3.3.0 (R Development Core Team, 2016).

Table VIII-1 Relationships between fight duration and RHP of contestants in different assessment strategies (Taylor and Elwood, 2003; Arnott and Elwood, 2009)

RHP parameter	Correlation between fight duration and RHP parameter		
	Pure self-assessment	Cumulative assessment	Mutual assessment
Loser RHP	+	+	+
Winner RHP	+	-	-
RHP difference	-	+	-
RHP average	+	+	N/A

Results

General Aggressive Behaviour Description

Aggressive interactions of *A. chelifer chelifer* stag beetles could be classified into four levels of aggressive intensity (Table VIII-2), i.e. level 0: no aggression, level 1: aggressive posture, level 2: one side-attack and level 3: wrestling. Aggressive behaviour of all contests occurred after physical contact and antennal touching on the opponent's bodies. From a total of 191 tested pairs, 118 contests were found to have aggressive behaviour (level 1, 2 or 3). The numbers of contests which had maximum level of aggression at level 1, 2 and 3 were 18, 20 and 80 contests, respectively. The main fighting styles was prying and lifting the opponents from the ground using their mandibles. Other fighting styles were also observed during the combat, such as pushing

and biting. Mandible interlocking was observed especially for the pairs with similar size. During wrestling, both males might stop their fight for several seconds and re-engaged again. From the total of 100 contests with fight (aggressive level 2 and 3), six contests could not be defined the outcome of fight because both males stopped the fight simultaneously, then retreated away from each other. Visible injuries in two males from two contests were observed, i.e. one male lost tarsi of a front leg and another lost tarsi of a hind leg, because their legs tightly grabbed the floor when the opponents were trying to pry and lift them.

Table VIII-2 Levels of aggression between male-male interactions of *A. chelifer chelifer*

Level	Performances	Description
0	no aggression	No aggressive action is observed.
1	aggressive posture	Male spreads mandibles wider or briefly twitches its head towards the opponent or uses mandibles to push the opponent. Attack or fight does not occur.
2	one side-attack	One male attacks, another male does not fight back but responds by retreat, running away, feigning death, or tightly clinging on the ground.
3	wrestling	Both males engage in fight.

Aggressive Intensity

Comparisons between aggressive level groups showed that average trait size of male pairs was significantly different in all observed traits (ML: $\chi^2_3 = 12.98$, $P = 0.004$; HL: $\chi^2_3 = 10.92$, $P = 0.012$; PL: $\chi^2_3 = 9.28$, $P = 0.026$; EL: $\chi^2_3 = 10.28$, $P = 0.016$; HW: $\chi^2_3 = 11.71$, $P = 0.008$; PW: $\chi^2_3 = 11.26$, $P = 0.010$) (Figure VIII-1). On the other hand, comparison between aggressive level groups using relative difference of trait size showed significant difference only in pronotum width (PW: $\chi^2_3 = 8.33$, $P = 0.039$) (Figure VIII-2).

Considering to the morph types, 96 males from the total of 115 major morph males and 12 males from the total of 19 minor morph males engaged in fights (aggressive level 2 or 3). Nevertheless, the ratios that major and minor morph males engaged in the fights were not significantly different ($\chi^2_1 = 3.10$, $P = 0.078$).

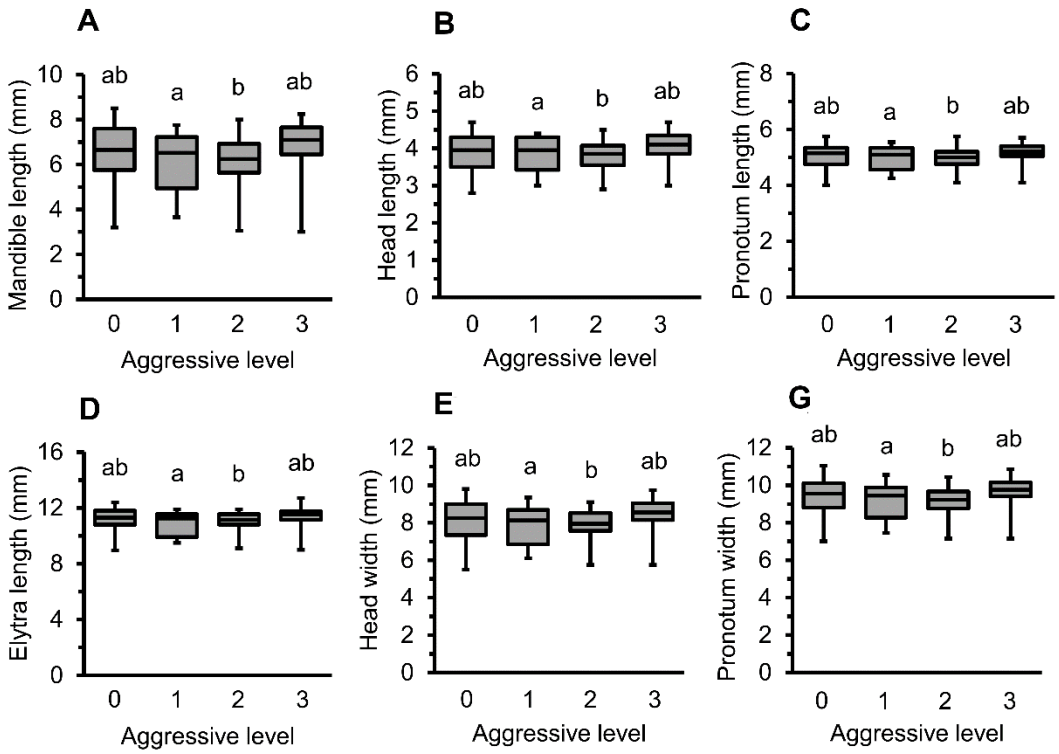

Figure VIII-1 Comparisons of (A) mandible length, (B) head length, (C) pronotum length, (D) elytra length, (E) head width and (F) pronotum width between aggressive level groups. A horizontal line within each box indicates the median, the box indicates 75 and 25 percentiles. Significant differences are denoted by different letters above the boxplots (Dunn's test: $P < 0.05$).

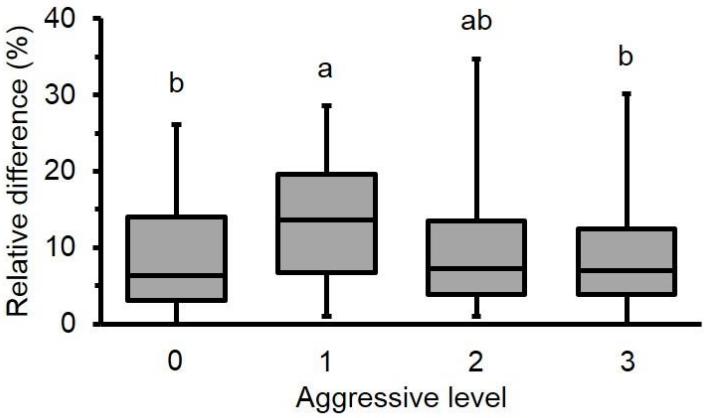

Figure VIII-2 Comparison of relative difference of pronotum width between different aggressive level groups. A horizontal line within each box indicates the median, the box indicates 75 and 25 percentiles. Significant differences are denoted by different letters above the boxplots (Dunn's test: $P < 0.05$).

Outcome of Fight

Only the contests with aggressive level 2 and 3 were analysed because the outcome of fights could be clearly defined. All morphological traits were significantly different between winners and losers which the winners were likely to possess larger traits (Table VIII-3). Final model of multiple logistic regression indicated that pronotum length, pronotum width and elytra length were important factors to the outcome of fights (Table VIII-4). Among these traits, pronotum width had the highest significance to the outcome of fights. Therefore, the pronotum width was assumed as the RHP of male *A. chelifer chelifer*. Probability of larger males (larger pronotum width) won the contests was higher than 50% when the RHP difference was greater than 2%, and nearly to 100% when the RHP difference was greater than 20% (Figure VIII-3).

Table VIII-3 Comparisons of morphological trait sizes between winners and losers of *A. chelifer chelifer* males

Morphological traits (n = 94)[a]	Median		*P*	Fight won by larger trait[b]
	Winner	Loser		
ML (mm)	7.6	6.5	< 0.001	72 (2)
HL (mm)	4.3	3.9	< 0.001	68 (5)
PL (mm)	5.35	5.0	< 0.001	66 (9)
EL (mm)	11.65	11.1	< 0.001	63 (10)
HW (mm)	9.0	8.0	< 0.001	73 (2)
PW (mm)	10.1	9.2	< 0.001	72 (2)

[a] The contests with aggressive intensity lower than level 2 and the contests which could not be identified the outcome of fight were excluded.

[b] Number in parentheses indicates the number of male-pair which had equal trait size.

Table VIII-4 Logistic regression analysis for morphological traits relating to the outcome of aggressive interactions between males of *A. chelifer chelifer*

Independent variables (n = 94)[a]	Coefficient	SE	Z value	*P*
Full model (AIC = 240.65)				
ML	-0.38	0.46	-0.82	0.414
HL	-0.28	0.90	-0.31	0.756
PL	-1.79	1.30	-1.37	0.171
EL	-0.83	0.48	-1.72	0.084
HW	0.58	0.85	0.68	0.497
PW	2.21	1.08	2.04	0.042
Constant	-3.68	4.22	-0.87	0.384
Final model (AIC = 235.57)				
PL	-1.87	1.24	-1.51	0.130
EL	-0.83	0.47	-1.76	0.078
PW	2.23	0.65	3.41	< 0.001
Constant	-2.28	3.10	-0.74	0.462

[a] The contests with aggressive intensity lower than level 2 and the contests which could not be identified the outcome of fight were excluded.

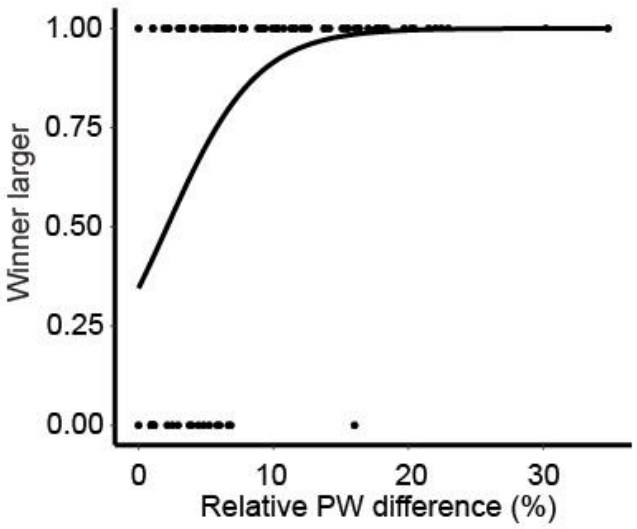

Figure VIII-3 Predicted probability that males of *A. chelifer chelifer* win contests based on relative RHP difference. The contests with aggressive intensity lower than level 2 and the contests which could not be defined the outcome of fight were excluded.

Initiation of Fight

From 100 testing pairs with fights (aggressive level 2 and 3), only 73 contests could be definitely defined whether which individuals attacked the opponents prior (initiator) or later (follower), while the other contests, two males started the fight at the same time. Male beetles which were initiators had morphological traits slightly smaller than the rivals, but it was not significantly different (Table VIII-5). There was no significant relationship between the initiators and the outcome of the fights ($\chi^2_1 = 2.31$, $P = 0.13$, n = 70; excluded the contests with aggressive intensity lower than level 2, the pairs which could not be identified the outcome of fights, and the pairs which two males began the fights at the same time).

Table VIII-5 Comparisons of morphological trait sizes between initiators and followers of *A. chelifer chelifer* males

Morphological trait (n = 73)[a]	Median		*P*
	Initiator	Follower	
ML (mm)	6.8	7.1	0.783
HL (mm)	3.9	4.1	0.937
PL (mm)	5.1	5.2	0.806
EL (mm)	11.4	11.5	0.697
HW (mm)	8.2	8.5	0.973
PW (mm)	9.4	9.7	0.984

[a] The contests with aggressive intensity lower than level 2 and the contests which two males began the fight at the same time were excluded.

Fight Duration

Only the contests with aggressive level 3 (80 contests) were analysed. Fight duration was greatly varied, ranging from 1 to 1,042 seconds. Duration of fight was negatively correlated with absolute RHP difference (r_s = -0.491, $P < 0.001$) and relative RHP difference (r_s = -0.487, $P < 0.001$), but it was not correlated with loser RHP (r_s = 0.173, P = 0.124), winner RHP (r_s = -0.189, P = 0.094) and average RHP of fight pairs (r_s = 0.025, P = 0.827) (Figure VIII-4). However, the relationships between RHP and fight duration were not consistent with any assessment strategies shown in Table VIII-1.

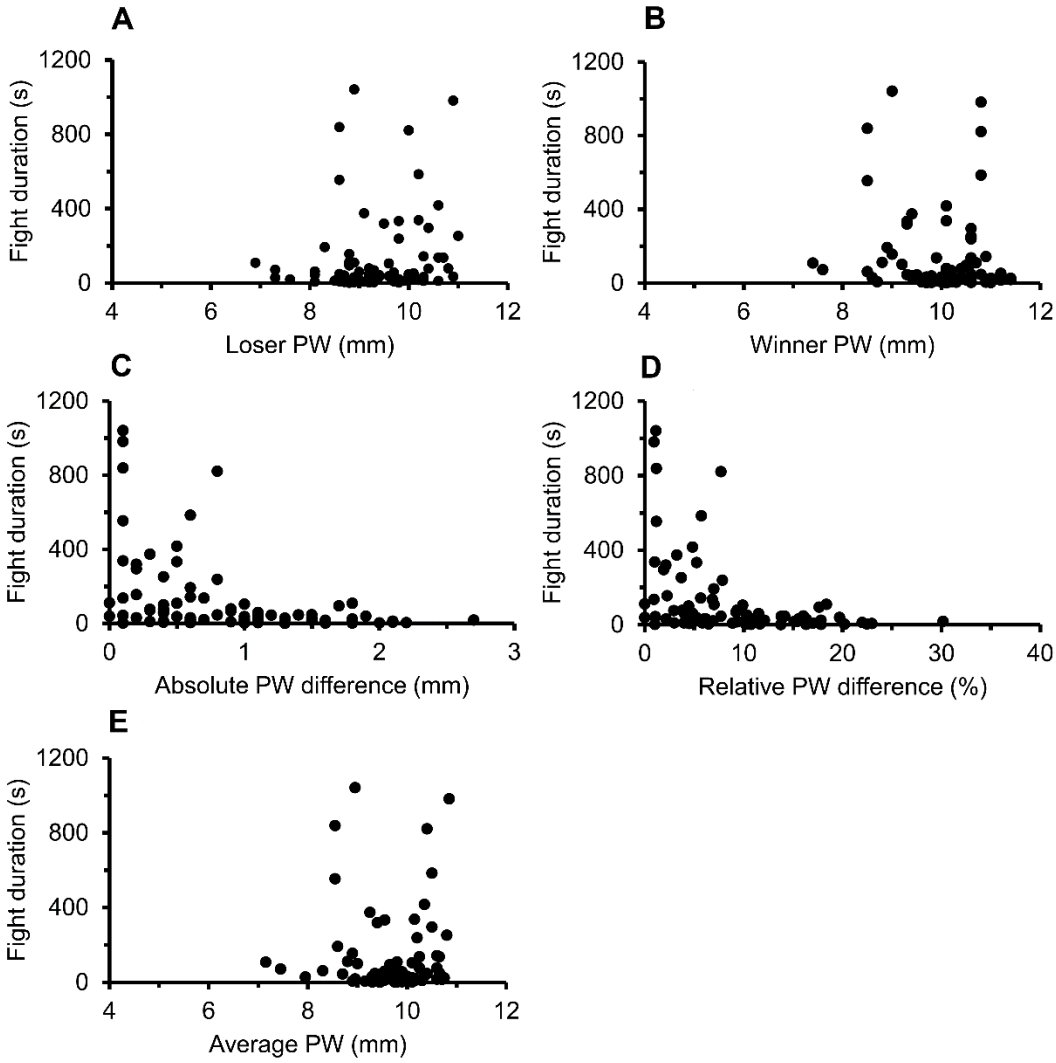

Figure VIII-4 Relationships between fight duration and (A) loser PW, (B) winner PW, (C) absolute PW difference, (D) relative PW difference and (E) average PW of *A. chelifer chelifer* males.

Discussion

One distinct behaviour of adult *A. chelifer chlifer* was feigning death when they were disturbed. This behaviour could last for several minutes before they continued other behaviours. Aggressive behaviour against the researchers was never observed. Normally, other male stag beetles often exhibit aggressive behaviour against the sources of disturbance not only conspecific males but also other stag beetle species, other insects and researchers, by showing aggressive posture and/or fighting back (Hongo and Okamoto, 2013; Hongo, 2014; Goyens et al., 2015b). From the observation, aggression between males always occurred after physical contact and antennal touching. Thus, *A. chelifer chelifer* stag beetles probably use chemical compounds on cuticle surface as chemical cue for recognition of conspecific members rather than using visual cue as reported in other stag beetles (Goyens et al., 2015b). As a result, these could explain why aggressive behaviour of male *A. chelifer chelifer* happened after physical contact and it may also influence the initiation of fight.

Although aggressive intensity of male *A. chelifer chelifer* could be classified, escalation of aggression as sequential were indistinctive. Study in *P. inclinatus* and *L. maculifemoratus* showed that they assessed the strength of competitors from the width of opened mandible before decision to fight (Hongo and Okamoto, 2013). In this study, males opened mandibles wider from aggressive level 1, but any behaviours close to the assessment between them were not found. Moreover, the relationship or trend between the size of morphological traits and the decision to escalate the aggressive intensity were not found, although average size of all observed traits and relative difference of pronotum width were significantly different among aggressive level groups. From

these, it seemed that male *A. chelifer chelifer* did not assess opponent's ability relative to their own before the decision to fight. The results of this study showed that there were not significant differences of morphological trait size between initiators and followers. This indicated that the traits of males relative to their opponents (larger or smaller) did not affect the initiation of fight. Actually, smaller males tended to escalate the aggression to higher intensity (level) more frequencies than initiation by larger males (but it was not significantly different), although they tended to be losers from these fights. To explain this circumstance, it is necessary to consider on the place where the fights take place. After males were firstly attacked by initiators, they briefly lost stability to hold the ground and were likely to flip over. Because the contests in this experiments were conducted in the plain surface, males could re-engage in the combat again as long as the fight was not terminated. Smaller males normally have inferior fighting ability and so they have lower chance to win in this situation. On the other hand, if the fight takes place at a site high from the ground or on vertical surface such as branches or tree trunks, initiators will be at an advantage because it would attack before the opponents are ready to respond. That can cause instability to hold the surface and then falling to the ground, therefore the smaller males have higher chance to win. However, observation in the fields is needed to prove this hypothesis.

For the fights between males of *A. chelifer chelifer*, those possessing larger observed traits won the fights more frequently than smaller males. Among tested morphological traits, multiple regression analysis revealed that the best model for prediction of fighting outcome consisted of the combination of pronotum length, elytra length and pronotum width. By using pronotum width as RHP, it showed the importance of body size as an important factor to the outcome of fight. Probability of

larger males won the fights was greater than 50% when the relative RHP difference was only 2%. These results indicated that body size were more important than weapon size for fights of *A. chelifer chelifer*. This differed from studies in some stag beetles, such as *C. metallifer* (Goyens et al., 2015b), and other animals, such as rhinoceros beetles and shore crabs, that weapon size determined the fighting outcome independently from other traits (Sneddon et al., 1997; Karino et al., 2005; Small et al., 2009). The importance of body parts to the fighting outcome is probably implies to the muscle mass of beetles, which directly relates to their strength and ability to defeat the opponents (Goyens et al., 2015b; Goyens et al., 2015a). Moreover, the less importance of weapon size to the outcome of fight in this species may explain about the static allometry and why the ratio between mandible length and elytra length decreases in large males (Songvorawit et al., 2017b).

Fighting beetles which have body and weapon size variations, such as rhinoceros beetles and horned dung beetles, small or minor males usually avoid physical contact with other males, resulting in the rare combat between these males (Siva-Jothy, 1987; Karino et al., 2005). The results of this study were inconsistent with those beetles. Minor males of *A. chelifer chelifer* engaged in fight regardless how large the opponents are, and some minor males also initiated the fight. Similar result was found in *P. inclinatus*, which fighting frequencies was not different between major and minor males (Inoue and Hasegawa, 2013).

Assessment strategy of the stag beetles could not be concluded because significant relationships were found only in absolute and relative pronotum width differences, and that was possible to be either mutual assessment or pure self-

assessment (Taylor and Elwood, 2003; Arnott and Elwood, 2009). However, if considered to the correlation coefficients (r_s) regardless the *P-value* of the correlation test, it is close to the mutual assessment. Mutual assessment has an advantage over other strategies because it can reduce the cost of fight. When the difference is large, smaller males will give up the fight sooner after they perceive about the relative RHP. The risk of injury can be prevented (Arnott and Elwood, 2009). Although long fight duration was observed in the fight pairs with similar size, detectable injuries was rarely found. In this study, the injuries were observed only in two contests, but these injuries were not caused by infliction of the opponents directly.

CHAPTER IX

GENERAL DISCUSSION AND CONCLUSION

Stag beetles and many insects in the superfamily Scarabaeoidea have been reported to have high degree of intraspecific variation in their morphological characteristics (Shiokawa and Iwahashi, 2000a; Kawano, 2002; Moczek, 2002; Kawano, 2003; Harvey et al., 2011a; Iguchi, 2013). Many studies in these beetles indicated that the variations were mainly due to environmental variance (Moczek, 1998; Shafiei et al., 2001; Moczek, 2002; Karino et al., 2004; Okada and Miyatake, 2010; Gotoh et al., 2011; Hardersen et al., 2011; Gotoh et al., 2014; Romiti et al., 2017). The survey in the natural habitat of this study revealed that the preference of stag beetles, including *A. chelifer chelifer*, on decaying wood was based on (1) physical properties, i.e. moderate decay class (Class II−IV) which had moderate wood hardness and water content, and (2) nutritional properties, i.e. high nitrogen content and fungal biomass (Chapter III). The selection of oviposition sites by stag beetles was likely to depend on both the log decaying stage (or hardness) to protect immature stages from natural enemies and its nutritional properties to enhance the larval performance. Therefore, this result was an evidence about the importance of food to their fitness regarding to survivability and reproductive success, and was possibly responsible for body size variation of stag beetles in natural habitats.

By using *A. chelifer chelifer* as a model on morphological variation, the investigation on morphological characteristics was firstly conducted. Allometric

relationship between natural log of elytra length and natural log of mandible length best fitted to the continuous piecewise model proposed by Kotiaho and Tomkins (2001), and males could be divided into major and minor morphs based on the allometry indicating dimorphism in males of this species (Chapter IV). The declining allometric slope in large males could limit the weapon size in very large individuals, and is believed to be caused by resource allocation during the development (Knell et al., 2004; McCullough et al., 2015).

It is well known that phenotype is the result of interaction between both genotype and environment (Scheiner, 1993; Emlen and Allen, 2003; Lewis et al., 2012; Tsuchiya et al., 2012). Thus, it is possible that genetic factors in part contribute to the body size of stag beetles. Study on the body size variation by comparing between the wild-caught and the breeding specimens and between Bangkok (urban) and Chanthaburi (forest) populations revealed that body size distribution of breeding specimens was less than wild specimens and the overlap of the body size distribution between populations was lower in the breeding beetles (Chapter V). By comparing to beetles from the captive breeding, the result demonstrated that body size variation of wild beetles was strongly affected by environmental variance in their natural habitats. Body size of Chanthaburi population stag beetles was significantly larger than of Bangkok population. Allometries were also significantly different between the two populations. Study on the larval performances showed similar relative growth rate, but male stag beetles of Chanthaburi population had longer feeding period, as a result they had more time to gain weight, therefore larger body size in the adults. The differences between the two populations could be explained by adaptation through larval performances and body size in order to respond to their habitats. However, narrow-

sense heritabilities (h^2) of the observed traits in adults were not significant. Although study within population showed no detectable heritability in the body size, this study indicated the possibility of genetic factors to play an important role on body size variation at interpopulation level.

This dissertation demontrated that nutrition, both diet quality and quantity, influenced the adult body size of stag beetles by regulating through the growth during larval stage (Chapter VI). The results was consistent with many studies in this beetle group that nutrition during larval stage was the major component responsible for phenotypic variation (Moczek, 1998; Shafiei et al., 2001; Moczek, 2002; Karino et al., 2004; Gotoh et al., 2011; Gotoh et al., 2014). Diet quantity had strong effects on adult body size variation through feeding and growth performances. The results also showed the ability of stag beetle larvae to adjust their feeding performances relying on the food supply, which was an evidence of adaptation to respond to resource limitation.

Studies on the effects of diet quality revealed that the decay of wood and nitrogen were important factors to the growth of stag beetle larvae (Chapter VII). Rearing with fermented sawdust resulted in better growth performances than rearing with non-fermented sawdust. This may be another reason to explain why stag beetle larvae in natural habitats are more frequently found in decaying logs than non-decaying logs (Araya, 1993b, a; Wood et al., 1996; Harvey et al., 2011a; Songvorawit et al., 2017a). For the effects of nitrogen, the best larval performances was obtained from the diet containing 0.50% nitrogen which was similar to the average nitrogen content in decaying logs with *A. chelifer chelifer* observed in the field (0.61 ± 0.15, see Appendix F). This result was consistent with the observation in other wood-feeding insects that

high nitrogen level in food could enhance the growth of insects (Ayres et al., 2000; Tanahashi et al., 2009). However, rearing with sawdust spawn of wood-decaying fungi resulted in adverse effects to stag beetles that lead to death before pupation. Most studies in other stag beetles indicated stag beetle larvae gained benefits from feeding on wood-decaying fungi colonizing in decaying wood and this information has been applied in the making commercial diets for rearing of stag beetle larvae (Wood et al., 1996; Ek-Amnuay, 2009; Tanahashi et al., 2009). From the observation in the field, fungal biomass in wood was a factor relating to wood preference and the average fungal biomass in logs contained *A. chelifer chelifer* stag beetles was 3.13 ± 0.28 (ranging from 2.43 to 8.50, see Appendix F), while the average fungal biomass in the artificial diets was 9.44 ± 1.09 (ranging from 5.01 to 13.96, see Chapter VII). Excessive fungal biomass may result in the reverse effects to stag beetles of this species.

However, it seemed that nitrogen level and other nutrients in the sawdust of rubber trees in this study were sufficient to enhance stag beetles close to optimizing growth without the necessity to add more supplements. Comparing to nutritional properties of logs containing stag beetles found in natural habitats and body size of wild stag beetles (Chapter III, V and Appendix F), nutritional properties of the rubber tree sawdust for making diets in this study were relatively inferior, but it still yielded adult beetles with average body size similar to the wild beetles. Furthermore, male body size variation from the experiment of diet quantity (Gini coefficient = 0.062, Chapter VI) was similar to the size variation in wild beetles (Gini coefficient = 0.065, Chapter V), while the size variation from the experiment of diet quality (Gini coefficient by combining males in all experiments = 0.038, Chapter VII) was distinctively lower (Figure IX-1). This indicated that diet quantity had more effects on body size variation

of *A. chelifer chelifer* than the diet quality. As a result, it could infer that body size variation of wild beetles is likely to be mainly affected by food availability or quantity rather than the nutritional properties in wood. It should be noted that other environmental factors, such as season, temperature and some stress, may influence to the morphological variation of stag beetles, but they were not included in this dissertation.

Body and weapon sizes were reported as essential component of fighting success in many beetles and also determined their aggression and behaviours during the competition (Karino et al., 2005; Hongo and Okamoto, 2013; Kijimoto et al., 2013; Hongo, 2014; Goyens et al., 2015b). However, both major and minor males of *A. chelifer chelifer* had aggression and engaged in fights with similar ratios. The analysis indicated that the size of body parts (pronotum length, pronotum width and elytra length) was more important than the size of weapon (mandible length) for fighting outcome. This result differed from other studies in stag beetles and other animals, that weapon size was more important (Sneddon et al., 1997; Karino et al., 2005; Small et al., 2009; Goyens et al., 2015b). Possible explanation may be related to the use of body parts as supporting traits during the fights (Goyens et al., 2015b; Goyens et al., 2015a). Moreover, the relatively less importance of weapon size to the outcome of fight in this species may explain about the decrease of the allometry in large males, that they tend to invest resources more during development in other crucial body parts than the weapon parts (Songvorawit et al., 2017b). By using pronotum width as proxy of resource holding potential (RHP), individuals possessing larger RHP won the fights more frequently than smaller ones. Fight duration positively correlated with the RHP

difference between two contestants. Of these, this study demonstrated the importance of size as an important role in male-male competition and sexual selection.

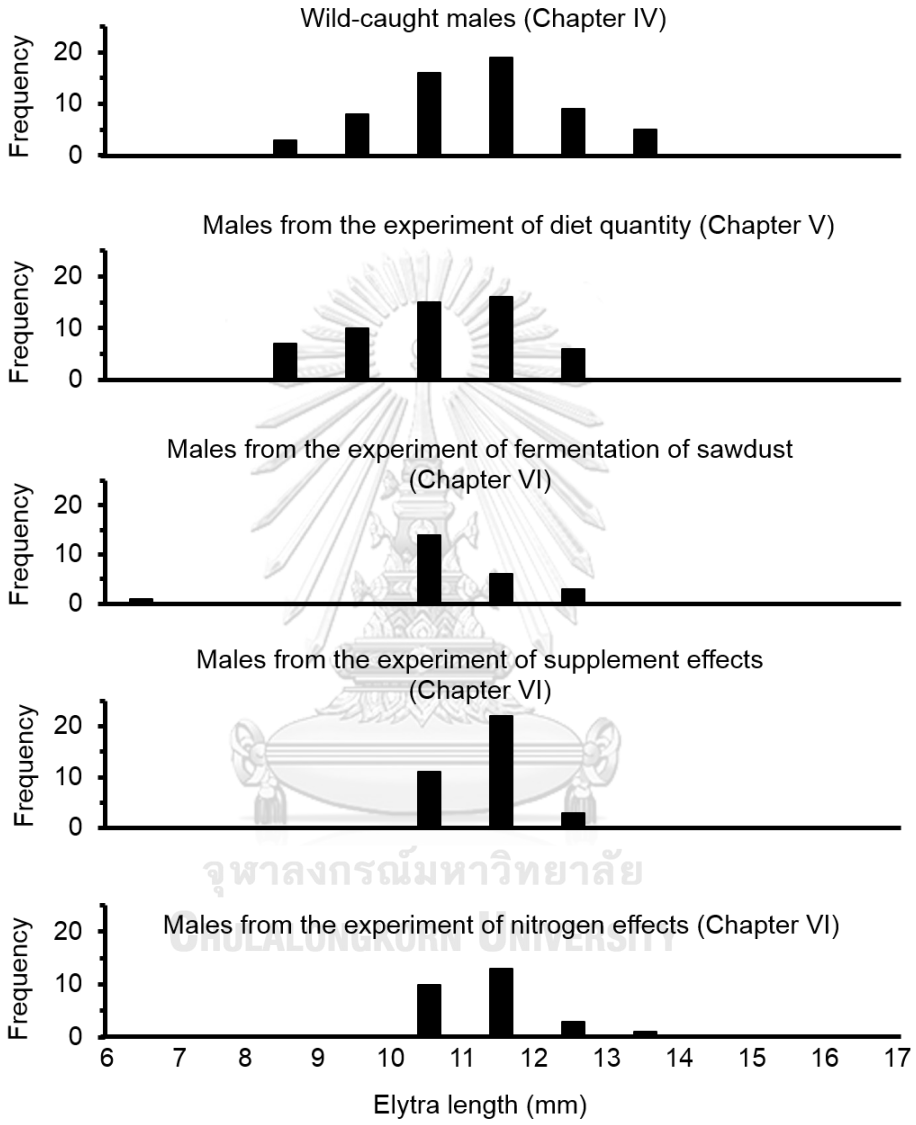

Figure IX-1 Body size variation of male *A. chelifer chelifer* stag beetles of Bangkok population from different experiment. Body sizes of males from the experiment of nitrogen effects were estimated from the relationship between maximal larval weight and adult body size obtained from Chapter V (elytra length = (maximal larval weight × 0.550) + 8.986).

A. chelifer chelifer seems to be one of the most successful stag beetle species to live in many habitats, including urban areas, and they require relatively low food quantity and quality to complete development (Chapters VI and VII). Furthermore, they also spend relatively shorter developmental time from eggs to adults (Chapters III and V). Therefore, study on digestive physiology and gut symbionts would help to clarify about the mode that they obtain nutrients, and that will fulfil the knowledge gap about adaptive strategies that help them to live in various habitats and overcome the problem of resource limitation. Additionally, this dissertation showed the possibility that body size difference between the two populations might be due to genotypic differences (Chapter V). Genetic effects on body size, therefore, would be another interesting topic in the future to know how genotype contributes to body size variation of stag beetles.

This dissertation indicated that decaying wood was the main factors for living of stag beetles. Therefore, any action plans for conservation or protection of stag beetles and other saproxylic insects should regard to dead trees as an important resource for maintaining these insect population. Moreover, study of morphological variation in stag beetles not only helps to improve understanding in the mechanism of the variation, but also can be used as a guideline to produce high quality beetles. Stag beetles from captive breeding will replace wild beetles in the markets and reduce capturing of beetles that is beneficial to the conservation of stag beetles.

In conclusion, this dissertation showed the importance of nutrition as a component of body size variation in stag beetles by regulating through feeding and growth performances during larval stage and further affect fighting ability and fighting outcome. Although effects of genetic factors on the body size were not proved in this

study, it is possible that body size difference between populations was due to genotypic difference (Figure IX-2).

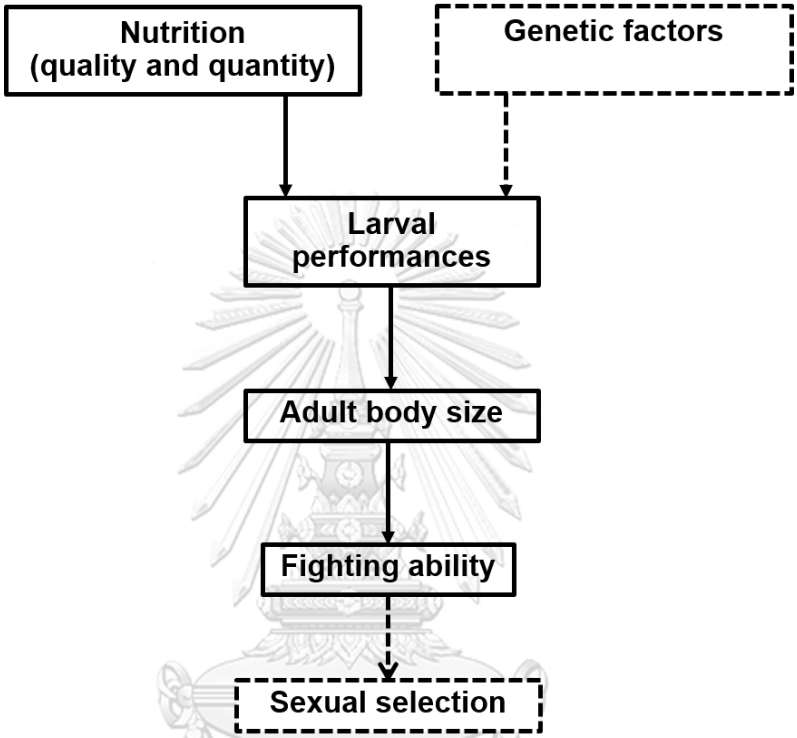

Figure IX-2 Diagram of the relationships between nutrition, body size and fighting ability of *A. chelifer chelifer* stag beetles. Dash boxes and lines represent other possible components of the relationships.

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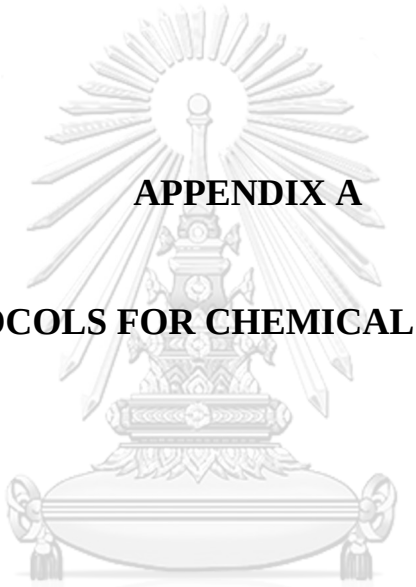
APPENDICES

APPENDIX A

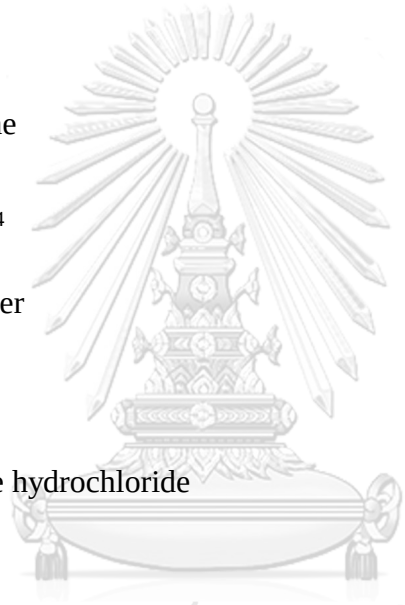
PROTOCOLS FOR CHEMICAL ANALYSES



Fungal Biomass Estimation

Chemicals

1. Para-dimethylaminobenzaldehyde
1. Conc. HCl
2. 95% ethanol
3. Na_2CO_3
4. Acetylacetone
5. Conc. H_2SO_4
6. Distilled water
7. NaOH
8. Glucosamine hydrochloride



Reagents

1. Ehrlich Reagent

Mix 1.6 g of para-dimethylaminobenzaldehyde in 30 ml of conc. HCl and 30 ml of 95% ethanol.

2. Acetylacetone Reagent

Mix 13.28 g of Na_2CO_3 and 4 ml of acetylacetone. Then, adjust the volume of the solution to 100 ml with distilled water.

Methods

1. Pretreatment of Samples

1.1 Add 2 ml of conc. H_2SO_4 to 0.5 g of dry weight wood samples, and leave for 24 h at room temperature.

1.2 Dilute the mixture to make 1 N H_2SO_4 (approximately 34 ml) with distilled water.

1.3 Heat in autoclave at 121 ºC for 1 h.

1.4 Neutralise with 1 N NaOH (approximately 34–36 ml) and adjust the volume to 100 ml with distilled water.

2. Glucosamine Measurement

2.1. Mix 1 ml of the sample solution with 1 ml of acetylacetone reagent and boil in water bath for 20 min.

2.2 Add 6 ml of 95% ethanol and 1 ml of Ehrlich reagent into the sample solution, and incubate at room temperature for 30 min.

2.3 Read absorbance in a spectrophotometer at 530 nm.

2.4 Compare concentration of glucosamine in the sample to the standard curve of glucosamine hydrochloride solution. Fungal biomass is expressed as glucosamine-equivalent in the unit of mg/g of wood dry weight.

Standard Solution

To prepare 100 mg/ml stock solution of glucosamine hydrochloride, dissolve 1.203 g of glucosamine hydrochloride in distilled water and adjust the volume to 10 ml, and then dilute to 0–24 mg/ml to make standard solutions (Table A-1 and Figure A-1). Perform the standard solutions with the same procedure as describe above (since the pretreatment step).

Table A-1 Preparation of glucosamine hydrochloride standard solutions

Tube	Glucosamine hydrochloride (ml)	Distilled water (ml)	Final concentration (mg/ml)
1	-	10	0
2	0.1	9.9	1.0
3	0.2	9.8	2.0
4	0.4	9.6	4.0
5	0.8	9.2	8.0
6	1.2	8.8	12.0
7	1.6	8.4	16.0
8	2.0	8.0	20.0
9	2.4	7.6	24.0

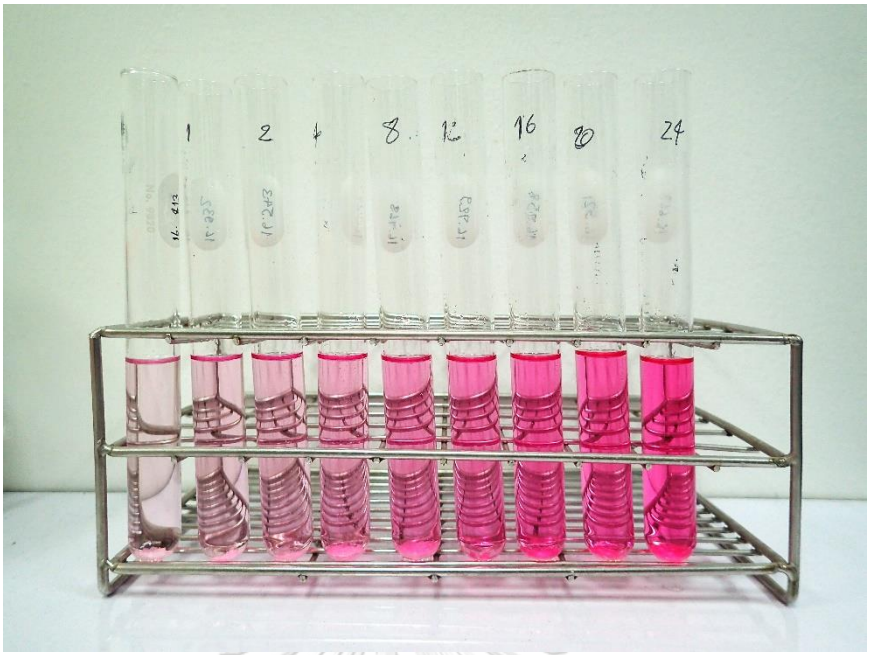


Figure A-1 Standard solutions of glucosamine hydrochloride.

Neutral Detergent Fibre and Neutral Detergent Soluble Analysis

Chemicals

1. Sodium dodecyl sulfate (sodium lauryl sulfate)
2. Triethylene glycol
3. $Na_2HPO_4.10H_2O$
4. Disodium ethylenediaminetetraacetate (EDTA) dehydrate
5. Na_2SO_3
6. α-amylase
7. $Na_2B_4O_7.10H_2O$ (Borax)
8. Distilled water

Neutral Detergent Fibre (NDF) Reagent

Mix 30 g sodium dodecyl sulfate, 10 ml triethylene glycol, 4.56 g Na_2HPO_4, 6.81 g $Na_2B_4O_7.10H_2O$ and 18.61 g disodium ethylenediaminetetraacetate (EDTA) dehydrate, then adjust volume to 1,000 ml with distilled water.

Methods

1. Add 1 g of dried ground sample and 0.5 g Na_2SO_3 into 50 ml of NDF reagent.
2. Boil the mixture for 5 min.
3. Add 0.1 ml of α-amylase and further heat for 60 min.

4. Weigh Whatman filter paper.

5. Filter the mixture (Figure A-2) through the Whatman filter paper and wash with hot distilled water 2 to 3 times and acetone 2 times.

6. Dry the remained fibre (NDF) in an oven at 100 ºC overnight and then weigh the NDF.

Calculation

Fibre weight (g) = Weight of filter paper with fibre – Weight of filter paper

NDF (%) = (Fibre weight – Sample dry weight) × 100

Neutral detergent soluble (NDS) (%) = 100 – NDF

Figure A-2 Wood samples after boiling in NDF reagent.

Total Phenol Analysis

Chemicals

1. 70% acetone

2. 1 N Folin-Ciocalteu reagent

3. 20% (w/v) Na_2CO_3: Dissolve 40 g of Na_2CO_3 in distilled water and adjust the volume to 200 ml.

Methods

1. Phenol Extraction

1.1 Add 200 mg of wood samples into 5 ml of 70% acetone, cool the mixture by keeping the tube on ice and shake for 10 min.

1.2 Centrifuge the mixture at 2500g at 4 °C for 10 min and then collect the supernatant.

1.3 Repeat the extraction for 2 times.

1.4 Pool the supernatant and adjust the volume to 10 ml by 70% acetone.

2. Phenol Measurement

2.1 Add 0.2 ml of phenol extracted solution into 0.8 ml distilled water.

2.2 Add 0.5 ml of 1 N Folin-Ciocateu reagent and then 2.5 ml of Na_2CO_3 solution.

2.3 Vortex the tube and allow colour development in the dark for 40 min.

2.4 Read absorbance in a spectrophotometer at 725 nm.

Standard Solution

Prepare 0.2 mg/ml stock solution of tannic acid by dissolving 1 g of tannic acid in distilled water and adjust the volume to 50 ml, then diluting the solution for 100-fold**.** Dilute the tannic acid solution to 0.00–0.20 mg/ml to make standard solutions (Table A-2). Perform the standard solutions with the same procedure as describe above.

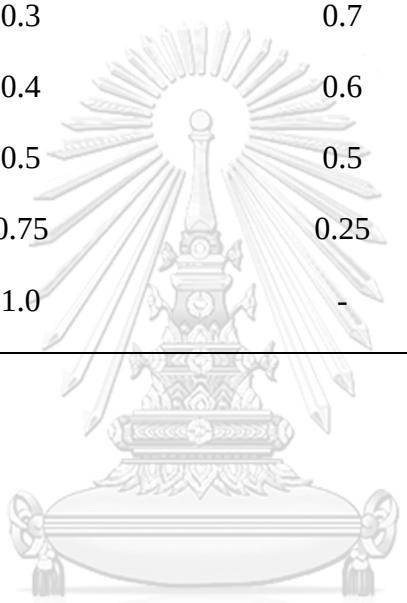
Calculation

Total phenols in the samples are expressed as tannic acid-equivalent. The amount of phenols is determined by comparison with standard curve of tannic acid solutions. Amount of phenols in a sample is calculated as the following equation:

Total phenols in sample (mg/g) = Measured concentration (mg/ml) × 50

Table A-2 Preparation of tannin acid standard solutions

Tube	Volume of tannic acid solution (ml)	Volume of distilled water (ml)	Final concentration (mg/ml)
1(blank)	-	1.0	0
2	0.1	0.9	0.02
3	0.2	0.8	0.04
4	0.3	0.7	0.06
5	0.4	0.6	0.08
6	0.5	0.5	0.10
7	0.75	0.25	0.15
8	1.0	-	0.20



Total Soluble Sugar Analysis

Chemicals

1. 80% ethanol
2. 2% phenol solution
3. Conc. H_2SO_4
4. Glucose
5. Fructose
6. Galactose

Methods

1. Sugar Extraction

1.1 Add 100 mg of wood samples into 5 ml of 80% ethanol, and then boil the mixture in water bath for 10 min.

1.2 Centrifuge the mixture at 2500g for 10 min and then collect the supernatant.

1.3 Repeat the extraction for 3 times.

1.4 Pool the supernatant and adjust volume to 15 ml by adding 80% ethanol.

2. Sugar Measurement

2.1 Mix 0.5 ml of sugar extracted solution with 1 ml of 2% phenol solution.

2.2 Rapidly add 2.5 ml of conc. H_2SO_4 and allow colour development in the dark for 30 min.

2.3 Read absorbance in a spectrophotometer at 490 nm.

Standard Solution

1. Prepare 10 mg/ml of glucose, fructose and galactose solutions by adding 0.5 g of sugar in 80% ethanol and adjust final volume to 50 ml.

2. Mix 1 ml of each sugar solution and adjust final volume to 30 ml using 80% ethanol. One mg/ml of sugar mixture will be obtained at this step.

3. Dilute the sugar mixture to 0–400 µg/ml to make standard solutions (Table A-3 and Figure A-3). Perform the standard solutions with the same procedure as describe above (since the sugar measurement method).

Calculation

Soluble sugar content in the samples is determined by comparison with standard curve of sugar mixture solutions. The measured concentration (µg/ml) is then transformed to the unit of mg/g of wood dry weight as the following equation:

Total soluble sugar (mg/g) = Measured concentration (µg/ml) × 0.15

Table A-3 Preparation of sugar mixture standard solutions

Tube	Volume of sugar mixture (ml)	Volume of 80% ethanol (ml)	Final concentration (µg/ml)
1	-	10.00	0
2	0.25	9.75	25
3	0.50	9.50	50
4	0.75	9.25	75
5	1.00	9.00	100
6	1.50	8.50	150
7	2.00	8.00	200
8	2.50	7.50	250

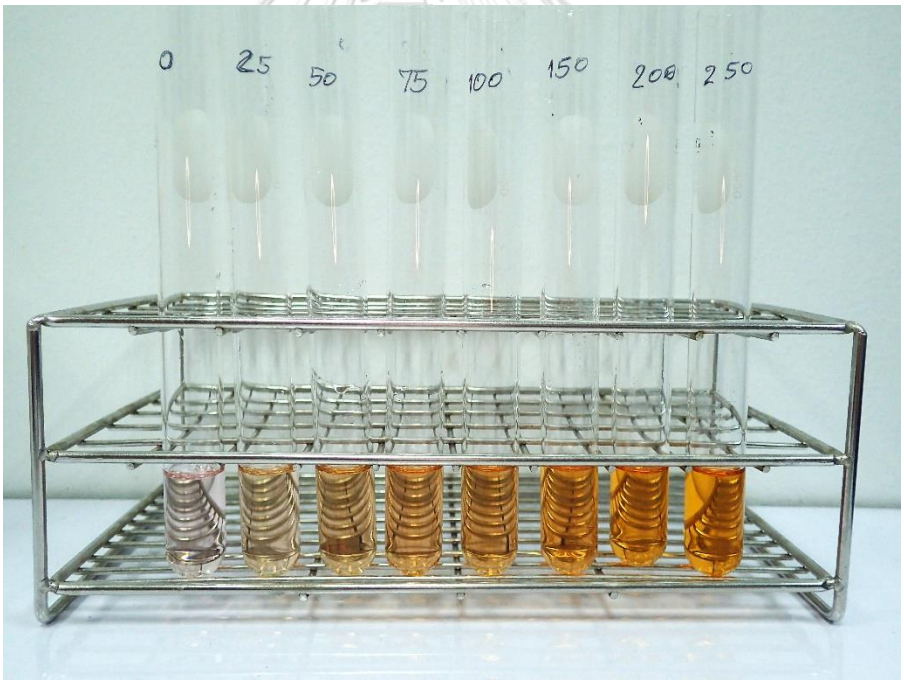


Figure A-3 Standard solutions of mixed sugars.

Chromic Oxide Analysis

Chemicals

1. 5.25% sodium hypochlorite
2. 1 M HCl
3. Distilled water

Methods

1. Sample Preparation

1.1 Dry the sample at 60 °C until constant weight.

1.2 Heat the sample at 500–600 °C for 8 h in order to ashing.

2. Chromium Measurement

2.1 Mix the ash sample and 2 ml of chlorine bleach in a test tube.

2.2 Heat the sample at 110 °C until the bleach is evaporated from the sample (approximately 15–20 min).

2.3 Add 10 ml of bleach and then heat further until the bleach is evaporated.

2.4 To remove excess hypochlorite, treat the sample in a similar manner but using 1 M HCl instead of bleach.

2.5 Add 10 ml distilled water into the test tube.

2.6 Read the optical density in spectrophotometer at 440 nm.

Standard Solution

1. Prepare 10 mg (tube 1) and 20 mg (tube 2) of Cr_2O_3 in glass tubes.
2. Treat the Cr_2O_3 in the same manner as the chromium measurement in samples but increasing all chemicals to 10x.
3. Aliquot the solution and dilute with distilled water following Table A-4.

Table A-4 Preparation of Cr_2O_3 standard solutions

Tube	Sample solution (ml)	Distilled water (ml)	Final amount of Cr_2O_3 (mg)
Blank	0	2	0
Tube 1	0.2	1.8	0.1
	0.4	1.6	0.2
	0.8	1.2	0.4
	1.6	0.4	0.8
Tube 2	1.2	0.8	1.2
	1.6	0.4	1.6
	2	0	2.0

APPENDIX B

CULTIVATION OF FUNGI, SCREENING AND IDENTIFICATION

Preparation of Culturing Media

2% Malt Extract Agar

Malt extract	20	g
Agar	15	g

Dissolve all components in 1000 ml of distilled water. Autoclave at 121 °C for 15 min.

2% Malt Extract Agar + Tetracycline

Malt extract	20	g
Agar	15	g
Tetracycline stock solution	2	ml

Dissolve malt extract and agar in 1000 ml of distilled water. Autoclave at 121 °C for 15 min. After autoclaving, aseptically add 2 ml of tetracycline stock solution into the medium to achieve final concentration of 50 mg/l before pouring.

2% Malt Extract Agar + Tetracycline + Carbendazim

Malt extract	20	g
Agar	15	g
Tetracycline stock solution	2	ml
Carbendazim stock solution	5	ml

Dissolve all components in 1000 ml of distilled water. Autoclave at 121 °C for 15 min. After autoclaving, aseptically add 2 ml of tetracycline and 5 ml of carbendazim stock solution into the medium to achieve both final concentrations of 50 mg/l before pouring.

Sorghum Grains for Making Spawn

Wash sorghum grains with tap water. Then, boil the grains at 100 °C for 10 min. Rinse water and air dry the grains approximately 6 h. Fill the grains in a glass bottle approximately a half of the total volume. Plug the bottle with cotton plug and autoclave at 121 °C for 30 min.

Carbendazim Stock Solution

Carbendazim 50% WP	0.4	g
70% ethanol	20	ml

Tetracycline Sock Solution

Tetracycline hydrochloride	0.5	g
Sterile distilled water	20	ml

Isolation of Wood-Decaying Fungi

This part is supporting information of Chapter VII. Pieces of wood or fruiting bodies from CWD with beetle larvae were surface-sterilised by soaking in 1% sodium hypochlorite for 10 sec and then rinsing with sterile water. Inner part of the samples (approximately 1 x 1 cm) was dissected and transferred onto agar plates. 2% malt extract agar supplemented with 50 mg/l tetracycline was used for general fungal isolation and 2% malt extract agar supplemented with 50 mg/l tetracycline and 50 mg/l carbendazim was used for isolation of basidiomycetes. The samples were incubated at room temperature for 2-5 days. Each isolates was purified until pure culture was obtained and maintained in 2% malt extract agar slant.

Screening of Wood-Decaying Fungi

This part is supporting information of Chapter VII. Screening was conducted based on extracellular enzyme production on agar plate. A single agar disc containing mycelium from edge of fungal colony was inoculated onto 4 screening agar medium, i.e. carboxymethylcellulose agar (CMC agar), xylan agar, ABTS agar and Azure B agar to characterised activities of 4 extracellular enzymes relate to wood component degradation, i.e. cellulose, xylanase, laccase and peroxidase. Brown-rot fungi were considered by positive result on CMC and xylan agar while white-rot fungi were positive result in CMC, xylan and ABTS and/or Azure B agar.

CMC Agar

One liter of the medium contain 0.5 g of $C_4H_{12}N_2O_6$, 1g of KH_2PO_4, 0.5 g of $MgSO_4.7H_2O$, 0.01 g of $CaCl_2$, 0.1 g of yeast extract, 5 g of CMC and 15 g of agar. After 3 days of incubation at 30 ºC, the plates will be flooded with 1% congo red and leaved for 15 min. The plates will be poured off, washed with distilled water and destained with 1M NaCl for 15 min. The presence of cellulase will be defined by a yellow-opaque area around the colony.

Xylan Agar

One liter of the medium contain 10 g of beechwood xylan, 5 g of peptone, 5 g of yeast extract, 1 g of K_2HPO_4, 0.2 g of $MgSO_4.7H_2O$, and 15 g of agar. After 3 days of incubation at 30 ºC, the plates will be flooded with iodine solution (0.25% w/v I_2 and KI) for 5 min. The plates will be poured off and washed with distilled. The presence of xylanase will be defined by a yellow-opaque area around the colony.

ABTS Agar

One liter of medium contain 0.25 g of ABTS, 0.5 g of $C_4H_{12}N_2O_6$, 1g of KH_2PO_4, 0.5 g of $MgSO_4.7H_2O$, 0.01 g of $CaCl_2$, 0.01 g of yeast extract, 2 g of glucose, 0.001 g of $CuSO_4.5H_2O$, 0.001 g of $Fe_2(SO_4)_3$, 0.001 g of $MnSO_4.H_2O$, and 16 g of agar. After 3 days of incubation at 30 ºC, the presence of laccase will be defined by green colour formation around the colony.

Azure B Agar

One liter of medium contain 0.1 g of Azure B, 0.5 g of $C_4H_{12}N_2O_6$, 1 g of KH_2PO_4, 0.5 g of $MgSO_4.7H_2O$, 0.01 g of $CaCl_2$, 0.01 g of yeast extract, 2 g of glucose, 0.001 g of $CuSO_4.5H_2O$, 0.001 g of $Fe_2(SO_4)_3$, 0.001 g of $MnSO_4.H_2O$, and 16 g of agar. After 3 days of incubation at 30 ºC, the presence of peroxidase will be defined by clearance of blue colour of the medium around the colony.

Identification of Fungi by Molecular Techniques

This part is supporting information of Chapter VII to show the procedure to identify *Daldinia eschscholtzii*

DNA Extraction

Genomic DNA was extracted from fresh mycelia using a modified CTAB method of Doyle and Doyle (1987)

1. Use a sterile spatula to scrape fungal mycelia from a culture plate into a microtube containing CTAB buffer (600 µl).
2. Grind mycelia using the microtube pestle.
3. Incubate the microtube at 65 °C for 20 min.
4. Add 600 µl of CHCl3:IAA (24:1), and invert repeatedly.
5. Centrifuge at 13,000 rpm for 5 min at 4 °C.
6. Remove the upper aqueous phase to a clean microtube.
7. Add 300 µl of cold isopropanol. Invert repeatedly and place at -20 °C for 20 min.
8. Centrifuge at 13,000 rpm for 15 min at 4 °C to pellet the DNA.
9. Discard supernatant. Add 50 µl of 1x TE to dissolve DNA pellet.

PCR: ITS

The internal transcribed spacer (ITS) region was amplified in a 50-ml reaction volume containing 1x buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM of each primer (ITS5 and ITS4), and 1 U Taq DNA polymerase. The PCR temperature profile began

with an initial denaturation at 96 °C for 2 min, followed by 35 cycles of 96 °C for 1 min, 53 °C for 1 min and 72 °C for 1:30 min. The final extension was carried out for 10 min at 72 °C.

Gel Electrophoresis and Sequencing

PCR product was checked by 0.8% agarose gel electrophoresis, stained with ethidium bromide, and visualised under UV transilluminator. The PCR product was sent to be sequenced for both directions on an automated DNA sequencer (Macrogen Inc., Korea).

Sequence Analysis

The nucleotide sequences obtained from all primers were assembled using Cap contig assembly program, an accessory application in BioEdit (Biological sequence alignment editor) Program (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html). The sequences were compared with nucleotide sequences databases on Genbank, CBS or suitable databases.

Nucleotide Sequence in ITS rDNA Region of *Daldinia eschscholtzii* (5' → 3')

CGTAACAAGGTCTCCGTTGGTGAACCAGCGGAGGGATCATTACTGAGTTA

TCTAAACTCCAACCCTATGTGAACTTACCGCCGTTGCCTCGGCGGGCCGCG

TTCGCCCTGTAGTTTACTACCTGGCGGCGCGCTACAGGCCCGCCGGTGGA

CTGCTAAACTCTGTTATATATACGTATCTCTGAATGCTTCAACTTAATAAG

TTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATCGAAGAACGC

AGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAA

TCTTTGAACGCACATTGCCCCCATTAGTATTCTAGTCGGCATGCCTGTTCG

AGCGTCATTTCAACCCTTAAGCCCCTGTTGCTTAGCGTTGGGAATCTAGGT

CTCCAGGGCCTAGTTCCCCAAAGTCATCGGCGGAGTCGGAGCGTACTCTC

AGCGTAGTAATACCATTCTCGCTTTTGCAGTAGCCCCGGCGGCTTGCCGTA

AAACCCCTATATCTTTACTCGTTGACCTCGAATCAGGTAGGAATACCCGCT

GAACTTAAGCATAT

Table B-1 Comparison of nucleotide sequences of *Daldinia eschscholtzii* with reference strains

	Description	Max score	Total score	Query cover	Ident	Accession
1	Uncultured fungus clone L042882-122-062-B11 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	1053	1053	100%	100%	GQ999459.1
2	*Daldinia eschscholtzii* culture-collection JMRC:SF:11930 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1048	1048	100%	99%	KU304335.1

Table B-1 (Continued)

	Description	Max score	Total score	Query cover	Ident	Accession
3	Fungal endophyte isolate 744 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1048	1048	100%	99%	KR016835.1
4	Uncultured fungus clone LX042400-122-057-C11 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	1048	1048	100%	99%	GQ999540.1
5	*Daldinia eschscholtzii* isolate BPEF73 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1042	1042	99%	99%	KF151849.1

Table B-1 (Continued)

	Description	Max score	Total score	Query cover	Ident	Accession
6	Uncultured fungus clone L042833-122-063-D09 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	1042	1042	100%	99%	GQ999495.1
7	Uncultured fungus clone L042882-122-062-C01 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	1042	1042	100%	99%	GQ999461.1
8	Uncultured fungus clone L042881-122-061-C05 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	1042	1042	100%	99%	GQ999441.1

Table B-1 (Continued)

	Description	Max score	Total score	Query cover	Ident	Accession
9	*Sporothrix* sp. STD57 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1042	1042	98%	100%	HM012821.1
10	Fungal sp. ARIZ B463 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1038	1038	100%	99%	FJ613058.1

Preparation of Sawdust Spawn

This part is supporting information of Chapter VII.

Methods

1. Prepare pure culture of fungus on malt extract agar plate (Figure B-1).
2. Inoculate the fungus into a bottle of sterile sorghum grains to make grain spawn.
3. Incubate at room temperature approximately 2-3 weeks.
4. After fungal mycelium fully grow in the sorghum grains (Figure B-2), inoculate the grain spawn into a cup containing sterile sawdust substrate approximately 20 grains per cup.
5. Incubate the cup at room temperature until the fungal mycelium fully grow in the cup (Figure B-3).

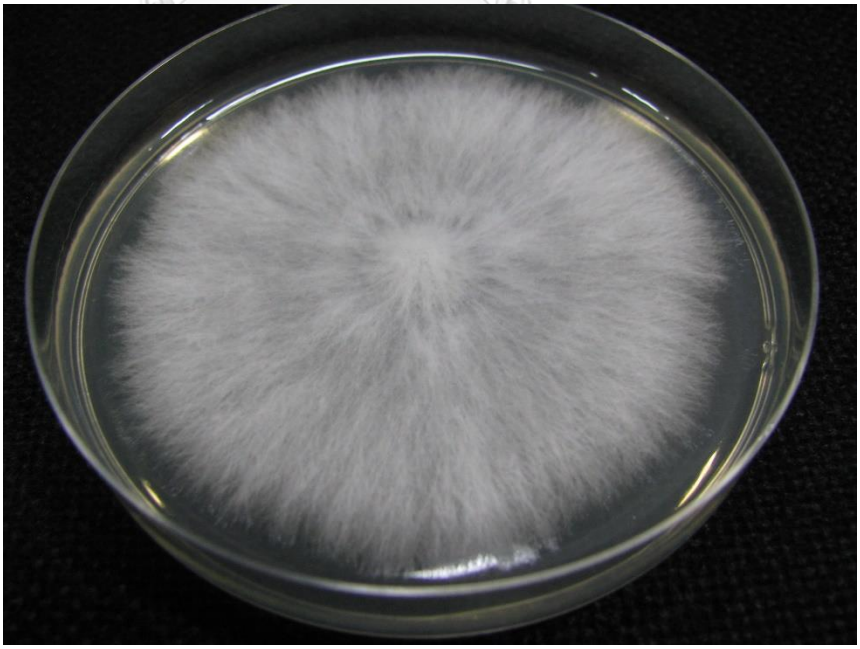


Figure B-1 Colony of wood-decaying fungus on malt extract agar.

Figure B-2 Sorghum grain spawn of wood-decaying fungi.

Figure B-3 Fungal mycelium in sawdust substrate.

APPENDIX C

SURVEY OF STAG BEETLES IN NATURAL HABITATS

Collecting Sites

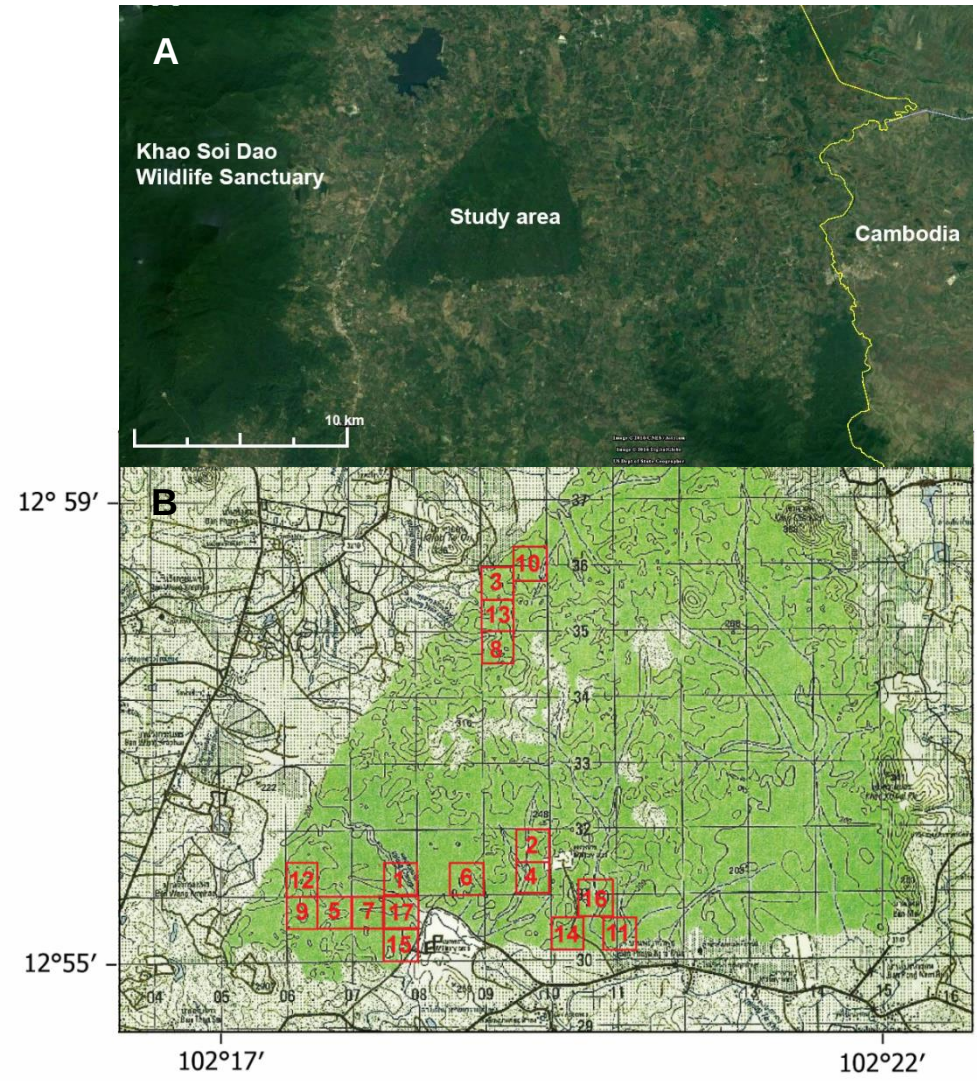


Figure C-1 (A) Satellite map of the study area in the dry-evergreen forest of Chanthuburi province (B) Collecting sites, red squares with number refer to the surveyed plots, each grid on the map equals to 1 km^2, numbers in the red squares refer to the date of survey (1 = July, 2013; 2 and 3 = September, 2013; 4 and 5 = November, 2013; 6 and 7 = January, 2014; 8 and 9 = March, 2014; 10 and 11 = May, 2014; 12 and 13 = July, 2014; 14 and 15 = September, 2014; 16 and 17 = November, 2014).

Figure C-2 Collecting sites in the dry-evergreen forest of the Marine's Paramilitary Task Force, Thewa Pitak Camp, Pong Nam Ron district, Chanthaburi province, Thailand.

Figure C-3 Collecting sites in Bangkok metropolitan area, Thailand**.**

Stag Beetle Collecting

Figure C-4 Procedure of stag beetle larvae sampling such as (A) measurement of log size, (B) decay class estimation, (C) breaking and (D) collecting.

Figure C-5 Stag beetle larvae found inside decaying logs.

Stag Beetles Found in Collecting Sites

Figure C-6 *Aegus chelifer chelifer* MacLeay, 1819.

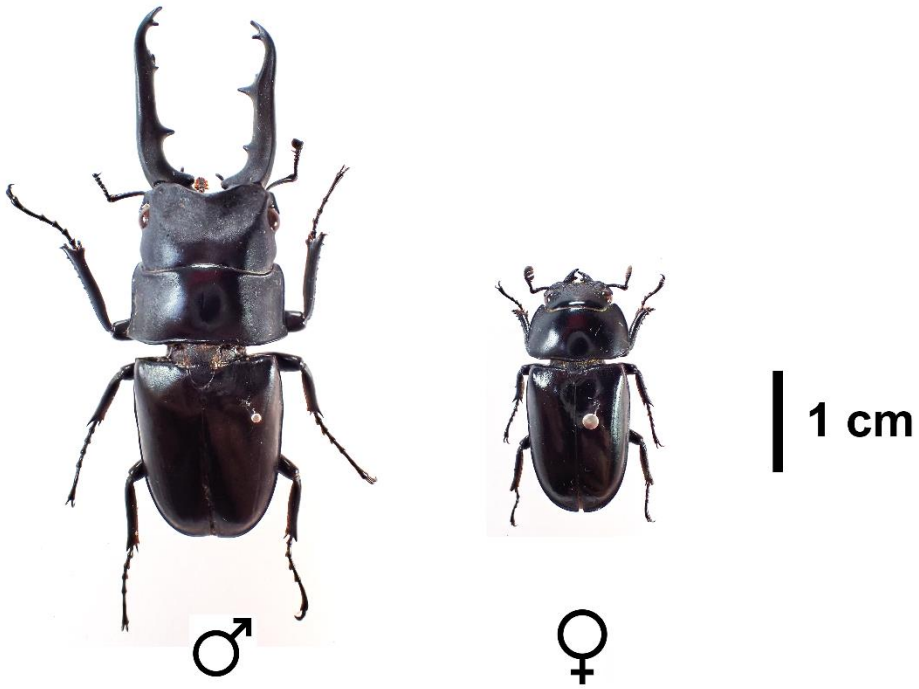


Figure C-7 *Prosopocoilus buddha* (Hope, 1842).

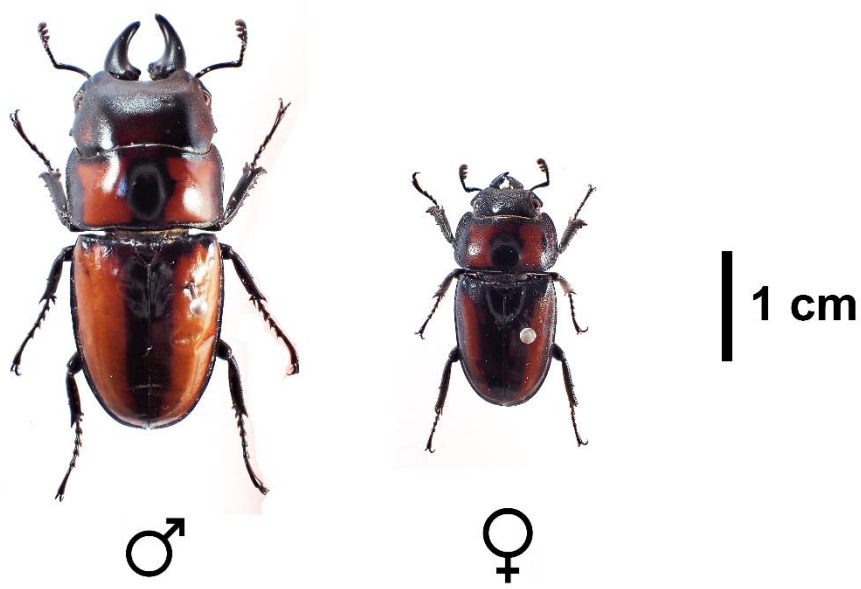


Figure C-8 *Prosopocoilus inquinatus nigripes* (Boileau, 1905).

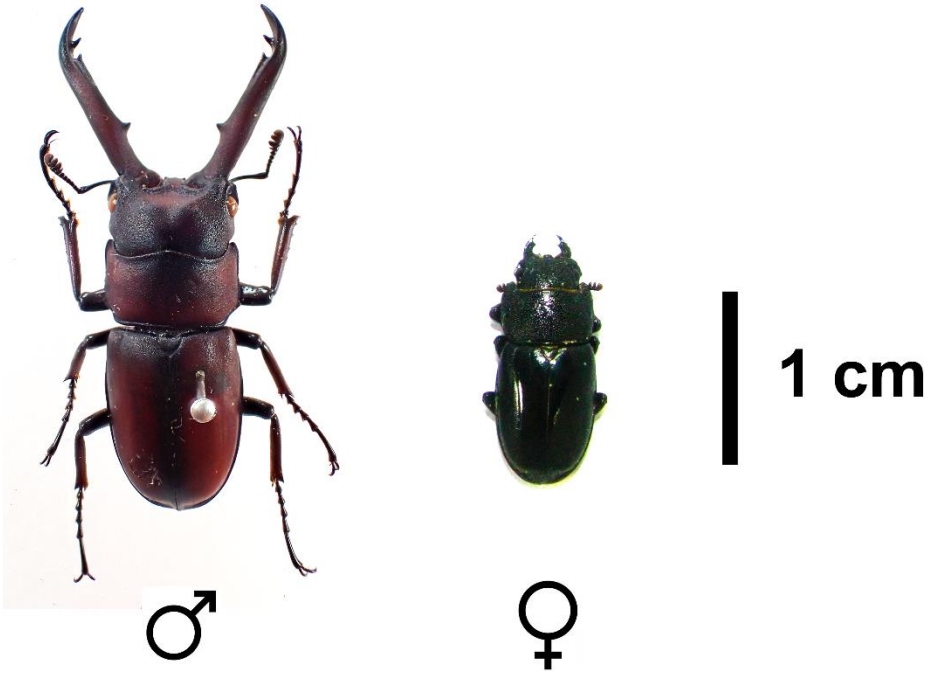


Figure C-9 *Prosopocoilus jenkinsi* (Westwood, 1848).

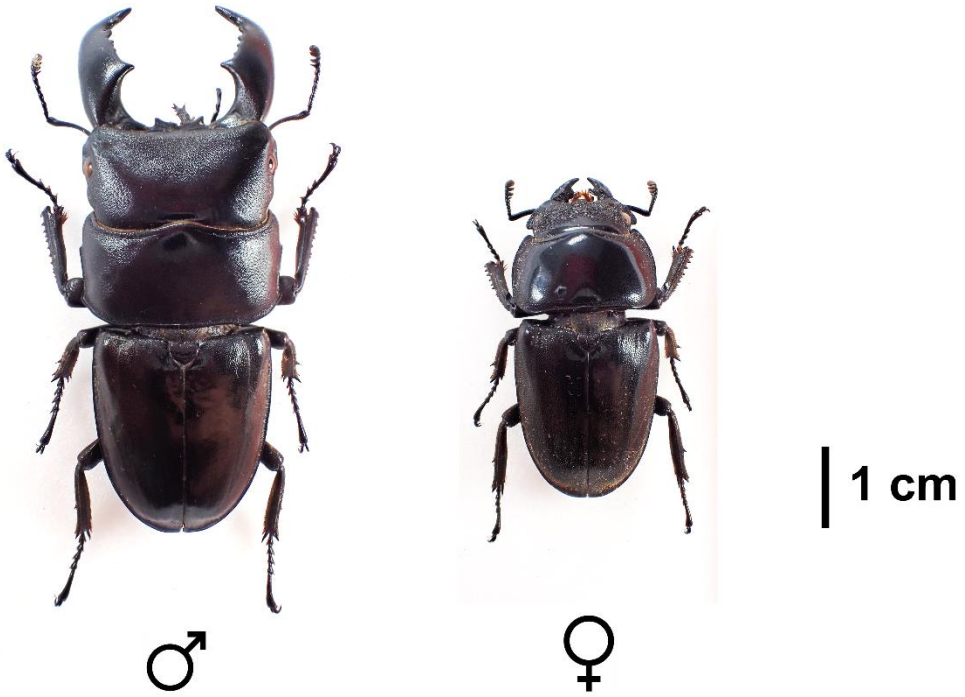


Figure C-10 *Dorcus titanus* (Boisduval, 1835).

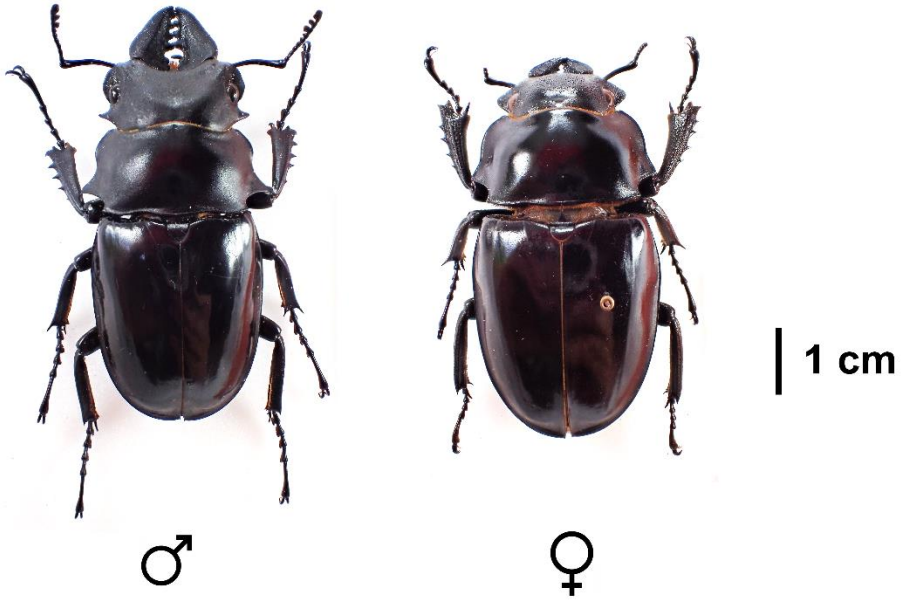


Figure C-11 *Odontolabis siva* (Hope & Westwood, 1845).

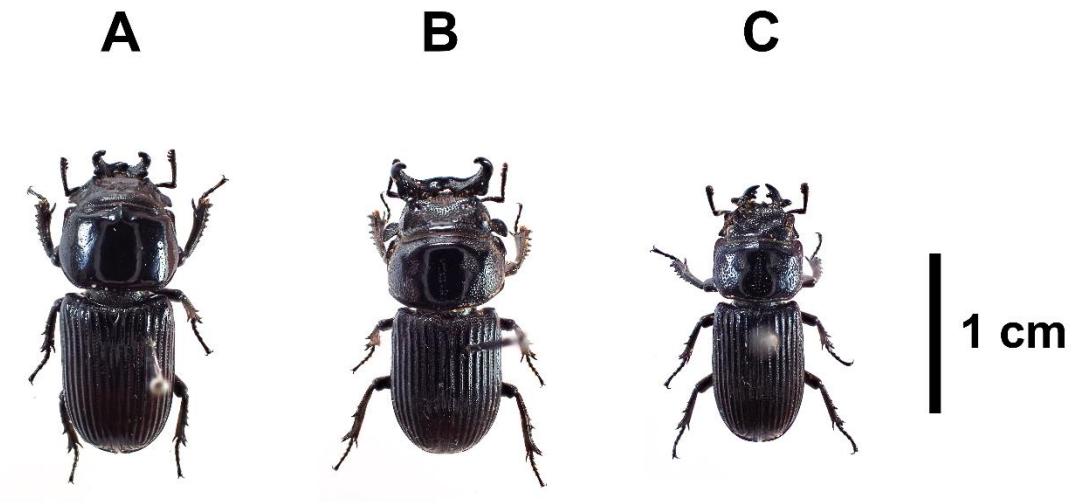


Figure C-12 (A) *Nigidius* sp. 1, (B) *Nigidius* sp. 2 and (C) *Nigidius distinctus* Parry, 1873 (found in a collecting site of Chanthaburi after field work has finished). Sex of these three species cannot be identified from external morphology.

APPENDIX D

STAG BEETLE REARING AND BEHAVIOURAL EXPERIMENT

Sawdust for Rearing of Stag Beetle Larvae

Sawdust is particles of wood which is a by-product of cutting or grinding logs. Sawdust in this disseartation belonged to the rubber tree, *Hevea brasiliensis* Mull. Arg. It was produced from a lumber mill in Rayong province, eastern Thailand. The quality of the sawdust was the same grade as the use for mushroom cultivation, which lacked contamination of other wood and chemicals or pesticides (Figure D-1). Sawdust in this study was obtained as fresh sawdust (age less than 7 days after processing in the lumber mill and had high moisture) and it was firstly process by sun drying approximately 5–7 days (Figure D-2). The dry sawdust was stored in containers at room temperature. Properties of the sawdust is shown in the Table D-1.

Table D-1 Data of sawdust used in this study

Property	Value
Price	3.50 baht/kg
Particle size	< 3 mm
Moisture of fresh sawdust	40–45%
Moisture after drying	< 10%
Temperature of fresh sawdust	45–50 °C
Temperature after drying	Room temperature
pH of fresh sawdust	9.3–9.5
pH after drying	7.0–7.5

Figure D-1 Sawdust of the rubber trees.

Figure D-2 Drying of fresh sawdust.

Figure D-3 Area for rearing of larval stag beetle *A. chelifer chelifer* locating at the terrace on 4th floor of Mahamakut Building, Chulalongkorn University. The largest boxes were breeding boxes (red arrow). The smaller boxes were used for experiments. The black plastic sheet at the shelves was used for prevent the light from the outside to make dark condition.

Figure D-4 (A) Rearing of adult stag beetle *A. chelifer chelifer* in 200 ml plastic cup containing moist tissue paper in the laboratory room and (B) feeding them with a piece of ripe banana which was replaced every 5 days.

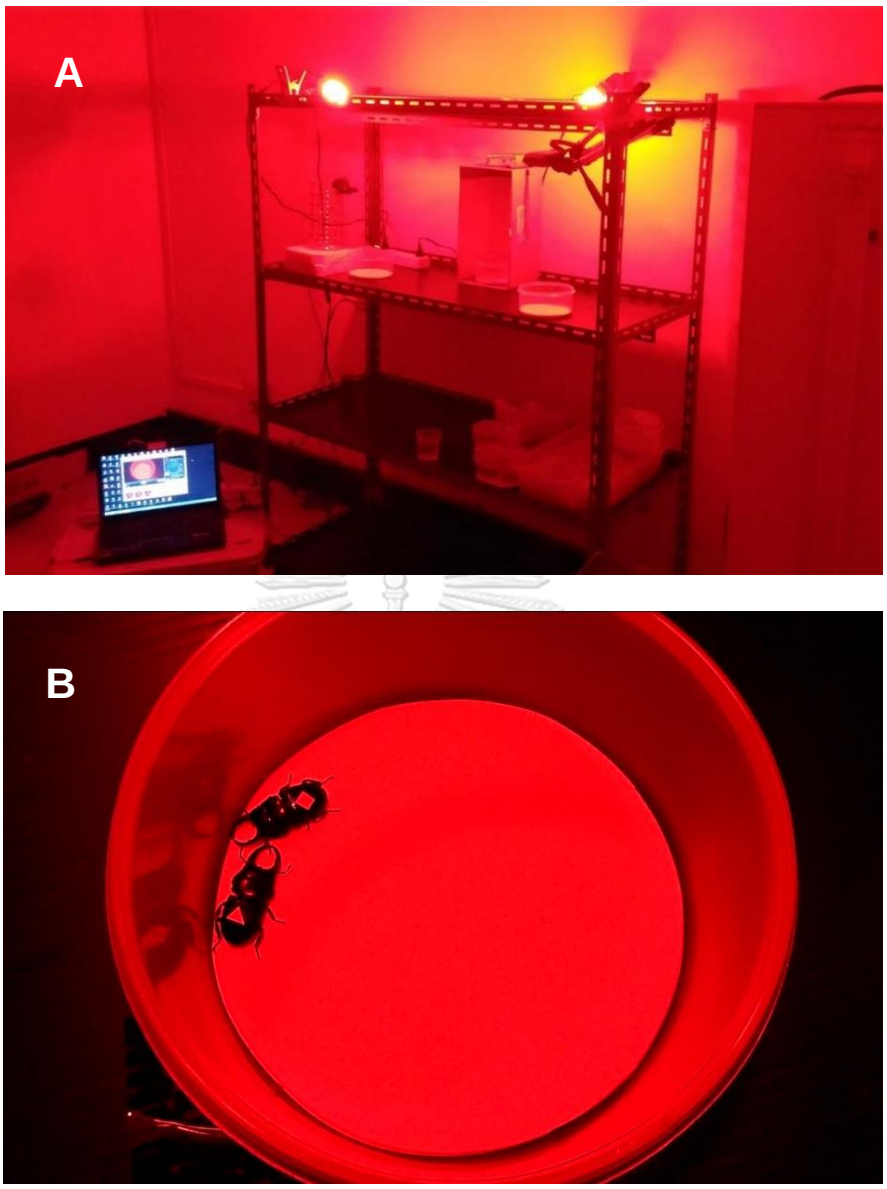


Figure D-5 (A) Laboratory room with red light for behavioural experiment and (B) male stag beetles in an arena made from round plastic container with the size of 15 cm in diameter and laid with EVA foam sheet at the bottom.

APPENDIX E

DATA OF SPECIMENS FROM MUSEUMS

Table E-1 Data of stag beetle specimens from museums

Specimen No.	Population	Sex	Museum/Collection site	Year	EL (mm)	ML (mm)
1	BKK	Male	DOA: Thon Buri, Bangkok	1946	12.2	8.1
2	BKK	Male	DOA: Bangkok	1937	10.4	5.5
3	BKK	Male	DOA: Bangkok Noi, Bangkok	1946	10.2	5.4
4	BKK	Male	DOA: Thanon Tok, Bang Kho Laem, Bangkok	1956	9.2	4.4
5	BKK	Male	DOA: Bangkok	1956	9	1.8
6	BKK	Male	DOA: Bang Khun Non, Bangkok Noi Bangkok	1948	10.6	5.7
7	BKK	Male	DOA: Chulalongkorn University, Bangkok	1955	11	6.8
8	BKK	Male	DOA: Department of Agriculture, Chatuchak, Bangkok	1949	10.3	5.8
9	BKK	Male	DOA: Thon Buri, Bangkok	1954	11.9	7.8
10	BKK	Male	DOA: Sathon, Bangkok	1956	9.8	4.9
11	BKK	Male	DOA: Bangkok	1956	11.7	7
12	BKK	Male	DOA: Thon Buri, Bangkok	1956	10.9	4.8
13	BKK	Male	DOA: Bang Khen, Bangkok	1957	11.3	5.9
14	BKK	Male	DOA: Phra Khanong, Bangkok	1957	10.5	6.5
15	BKK	Male	DOA: Bangkok	1957	9.5	4.9

BKK = Bangkok metropolitan area, CTI = Chanthaburi province, DOA = Insect Museum Thailand, Department of Agriculture, CU = Chulalongkorn University Museum of Natural History, FERC-3 = Forest Entomology Research Center 3-Chanthaburi, HMK = His Majesty the King Insect Park, EL = elytra length, ML = mandible length.

Table E-1 (Continued)

Specimen No.	Population	Sex	Museum/Collection site	Year	EL (mm)	ML (mm)
16	BKK	Male	DOA: Thon Buri, Bangkok	1957	11.7	8.4
17	BKK	Male	DOA: Royal Forest Department, Chatuchak, Bangkok	1960	9.7	6.3
18	BKK	Male	DOA: Bangkok	1953	13.2	7.8
19	BKK	Male	DOA: Bangkok	1953	12	8
20	BKK	Male	CU: Bangkok	1969	10.6	7
21	BKK	Male	CU: Thon Buri, Bangkok	1966	9.2	4.9
22	BKK	Male	CU: Pathum Wan, Bangkok	1955	10.2	5.7
23	BKK	Male	CU: Dusit, Bangkok	1956	9.1	3
24	BKK	Male	CU: Bangkok	1967	9	2.6
25	BKK	Female	DOA: Nonthaburi	1957	9.2	-
26	BKK	Female	DOA: Lumpini, Bangkok	1957	11.9	-
27	BKK	Female	DOA: Bangkok Noi, Bangkok	1946	10.9	-
28	BKK	Female	DOA: Bangkok	1954	10.7	-
29	BKK	Female	DOA: Bang Khen, Bangkok	1956	9.9	-
30	BKK	Female	DOA: Bang Khen, Bangkok	1956	10.1	-

Table E-1 (Continued)

Specimen No.	Population	Sex	Museum/Collection site	Year	EL (mm)	ML (mm)
31	BKK	Female	DOA: Thon Buri, Bangkok	1956	11.1	-
32	BKK	Female	DOA: Thon Buri, Bangkok	1958	8.2	-
33	BKK	Female	DOA: Bangkok	1959	10.2	-
34	BKK	Female	DOA: Pathum Wan, Bangkok	1955	10.8	-
35	BKK	Female	DOA: Bangkok	1958	10.4	-
36	BKK	Female	CU: Bang Yi Khan, Bangkok	1969	11.5	-
37	BKK	Female	CU: Pathum Wan, Bangkok	1955	11.4	-
38	BKK	Female	CU: Lumpini, Bangkok	1957	11.2	-
39	BKK	Female	CU: Thon Buri, Bangkok	1969	10.8	-
40	BKK	Female	CU: Bang Chak, Bangkok	1969	9.8	-
41	BKK	Female	CU: Bangkok	1968	10.4	-
42	BKK	Female	CU: Bang Sue, Bangkok	1971	11.9	-
43	CTI	Male	FERC-3: Pong Nam Ron, Chanthaburi	2013	10.2	6.4
44	CTI	Male	FERC-3: Pong Nam Ron, Chanthaburi	2008	11.2	6.7
45	CTI	Male	FERC-3: Pong Nam Ron, Chanthaburi	2008	10.1	6.1

Table E-1 (Continued)

Specimen No.	Population	Sex	Museum/Collection site	Year	EL (mm)	ML (mm)
46	CTI	Male	FERC-3: Pong Nam Ron, Chanthaburi	2008	11	4.9
47	CTI	Male	FERC-3: Pong Nam Ron, Chanthaburi	Unknown	11.6	6.9
48	CTI	Male	FERC-3: Pong Nam Ron, Chanthaburi	Unknown	12.5	7.3
49	CTI	Male	FERC-3: Pong Nam Ron, Chanthaburi	1993	11.2	6.1
50	CTI	Male	FERC-3: Pong Nam Ron, Chanthaburi	1993	11.2	6.8
51	CTI	Male	FERC-3: Pong Nam Ron, Chanthaburi	2012	13.6	7.1
52	CTI	Male	FERC-3: Pong Nam Ron, Chanthaburi	2004	13.8	9.1
53	CTI	Male	FERC-3: Pong Nam Ron, Chanthaburi	Unknown	12.7	8
54	CTI	Male	FERC-3: Khao Khitchakut National Park, Chanthaburi	2006	14.6	8.7
55	CTI	Male	FERC-3: Pong Nam Ron, Chanthaburi	1993	11.9	7
56	CTI	Male	FERC-3: Pong Nam Ron, Chanthaburi	2014	12.1	7.5
57	CTI	Male	FERC-3: Pong Nam Ron, Chanthaburi	2014	9	2.5
58	CTI	Male	FERC-3: Pong Nam Ron, Chanthaburi	1994	14.4	8.7
59	CTI	Male	CU: Chanthaburi	1957	11.5	6.6
60	CTI	Male	CU: Khao Soi Dao Wildlife Sanctuary, Chanthaburi	1973	8.9	2.7

Table E-1 (Continued)

Specimen No.	Population	Sex	Museum/Collection site	Year	EL (mm)	ML (mm)
61	CTI	Male	DOA: Chanthaburi	1969	9.5	3.3
62	CTI	Male	DOA: Chanthaburi	1967	9.7	4.2
63	CTI	Male	DOA: Chanthaburi	1967	9.1	3.6
64	CTI	Male	DOA: Chanthaburi	1959	14	9.5
65	CTI	Male	DOA: Chanthaburi	1955	10.8	7.9
66	CTI	Male	DOA: Laem Sing, Chanthaburi	1955	12.8	7.7
67	CTI	Male	HMK: Chanthaburi	2002	10.9	7.1
68	CTI	Female	DOA: Chanthaburi	1937	12.4	-
69	CTI	Female	DOA: Chanthaburi	1967	10	-
70	CTI	Female	DOA: Chanthaburi	1967	11.5	-
71	CTI	Female	DOA: Chanthaburi	1967	11.4	-
72	CTI	Female	DOA: Pong Nam Ron, Chanthaburi	1967	9.7	-

APPENDIX F

LOGS AND WOOD PROPERTIES

Table F-1 Properties of logs and wood collected from a dry-evergreen forest, Chanthaburi province, Thailand

Log No.	DC	Dn	Di	WA	WC	pH	P	NDS	SS	F	N	C/N	Occurrence/ Species
1	2	0.17	13.7	81.92	83.88	7.4	0.24	0.80	0.73	2.37	0.46	68.37	No
2	2	0.17	28	78.27	48.8	5.7	1.52	25.30	8.88	0.82	0.2	222.90	No
3	2	0.18	29	79.53	36.3	7.1	0.84	24.20	1.46	3.85	0.38	33.53	Unknown
4	2	0.20	28	75.40	77.55	6.2	0.55	15.20	4.04	2.48	0.66	64.92	PrIn
5	2	0.22	15.9	84.47	57.75	7	0.13	15.00	1.25	2.44	0.41	22.71	AeCh
6	2	0.23	14	70.78	66.12	6.5	0.72	20.80	6.67	5.03	0.8	37.23	AeCh, NiSp1
7	2	0.24	19.7	67.99	61.29	5.8	7.92	53.50	26.46	0.88	0.51	87.67	AeCh
8	2	0.25	17.5	62.85	45.24	6.5	1.51	4.00	9.36	1.72	0.28	161.46	No
9	2	0.26	12.7	74.98	63.05	6.3	1.18	1.01	9.83	2.90	0.44	96.95	No
10	2	0.26	41.4	69.98	52.46	6.4	3.35	20.89	9.74	2.93	0.51	83.78	AeCh

Wood properties: DC = Decay class; Dn = wood density (g/cm^3); Di = diameter (cm); WA = water absorption capacity; WC = water content (%); P = total phenol (mg/g); NDS = neutral detergent fibre (%); SS = total soluble sugar (mg/g); F = fungal biomass (mg/g); N = nitrogen content (%); C_N = carton to nitrogen ratio

Stag beetle species: AeCh = *Aegus chelifer chelifer*, PrBu = *Prosopocoilus buddha*, PrIn = *Prosopocoilus inquinatus*, PrJe = *Prosopocoilus jenkinsi*, DoTi = *Dorcus titanus*, OdSi = *Odontolabis siva*, NiSp1 = *Nigidius* sp.1, NiSp2 = *Nigidius* sp.2.

No = no stag beetle

Note: Randomly selected 78 logs were completely tested for all nutritional variables.

Table F-1 (Continued)

Log No.	DC	Dn	Di	WA	WC	pH	P	NDS	SS	F	N	C/N	Occurrence/ Species
11	2	0.32	11.5	61.03	25.34	6.5	5.01	16.60	6.20	4.43	0.8	50.61	NiSp1
12	2	0.35	19.7	56.87	20.06	5.2	23.23	38.50	46.09	1.99	0.33	148.03	No
13	2	0.36	12.1	52.72	40.74	6.8	0.34	21.10	1.94	3.73	0.6	42.62	AeCh
14	2	0.39	20.7	62.98	55.85	6.4	0.31	35.50	3.67	5.41	0.82	42.29	AeCh
15	3	0.09	50.9	86.65	68.47	5.7	0.50	19.30	5.09	1.31	0.35	124.40	Unknown
16	3	0.12	22.3	89.64	82.25	7.6	0.29	11.00	4.88	1.99	0.55	76.47	PrBu
17	3	0.14	21	69.14	50.54	7.1	0.47	18.90	3.94	1.81	0.37	117.41	PrBu
18	3	0.15	15.3	72.25	64.67	5.8	0.84	17.50	4.31	2.44	0.34	75.50	AeCh, PrJe
19	3	0.15	43.3	79.37	73.51	5.2	1.70	28.00	17.62	2.11	0.6	67.73	OdSi
20	3	0.16	11.1	69.86	76.17	7	0.79	10.70	4.99	2.43	0.45	91.60	PrBu
21	3	0.17	12	77.55	67.06	6.3	0.64	35.30	4.94	3.22	0.81	38.77	AeCh
22	3	0.17	16.6	75.81	66.28	5	1.14	15.20	10.73	1.67	0.24	168.67	No
23	3	0.17	26.1	60.06	44.87	6.4	1.69	20.40	6.88	2.48	0.46	102.33	No
24	3	0.18	29.9	57.51	61.03	6.4	0.30	9.70	4.88	2.34	0.29	159.90	No
25	3	0.19	16.9	77.86	46.32	6.6	0.72	7.00	5.94	2.27	0.34	140.38	No

Table F-1 (Continued)

Log No.	DC	Dn	Di	WA	WC	pH	P	NDS	SS	F	N	C/N	Occurrence/ Species
26	3	0.19	28.3	76.33	76	7	0.49	3.30	3.99	1.16	0.54	81.48	PrBu
27	3	0.19	26.7	77.22	67.72	6.2	0.67	5.10	4.62	0.48	0.18	249.39	No
28	3	0.19	15	42.17	33.77	6.2	3.39	67.70	19.15	3.40	0.63	74.43	NiSp1
29	3	0.19	19.1	72.70	79.4	6.2	0.80	21.60	10.67	2.23	0.78	48.73	AeCh
30	3	0.19	21	74.30	74.87	6.2	1.61	32.50	7.67	8.50	1.03	42.18	AeCh
31	3	0.20	11.8	69.48	65.58	7	0.41	19.70	4.57	2.07	0.5	82.00	PrBu
32	3	0.21	29.9	76.70	51.16	7.5	0.43	2.20	3.99	1.74	0.32	63.28	DoTi, PrIn
33	3	0.23	26.7	75.33	82.86	7	0.19	6.80	1.99	2.79	0.49	51.94	AeCh
34	3	0.23	21.6	70.25	51.27	5.6	7.43	6.20	11.78	1.71	0.37	122.19	No
35	3	0.23	17.5	70.05	64.95	6	0.55	29.10	2.73	2.35	0.79	51.10	AeCh, PrIn
36	3	0.25	33.7	73.98	64.55	7	0.18	22.50	3.04	2.41	0.46	59.02	AeCh
37	3	0.26	15	68.87	58.12	5.5	3.80	18.40	9.25	1.91	0.49	95.18	No
38	3	0.26	15.9	78.42	47.84	7	0.69	25.70	6.31	2.76	0.93	42.05	Unknown
39	3	0.27	15.6	71.22	73.72	6.7	1.40	3.80	3.62	3.35	0.76	51.79	AeCh, PrBu
40	3	0.28	33.1	69.15	68.09	6.9	4.01	19.00	11.67	0.71	0.38	120.11	PrJe

Table F-1 (Continued)

Log No.	DC	Dn	Di	WA	WC	pH	P	NDS	SS	F	N	C/N	Occurrence/ Species
41	3	0.35	13.7	62.20	71.91	6.5	0.66	19.00	11.31	3.57	1.15	34.41	AeCh, PrJe
42	4	0.10	12.1	83.52	81.58	7	0.52	8.60	4.31	4.03	0.69	56.77	Unknown
43	4	0.11	17.8	87.16	78.74	6.9	0.48	19.20	0.67	3.20	0.47	79.68	AeCh
44	4	0.11	22.3	80.33	67.37	6.9	0.85	28.10	4.73	5.73	1.22	35.49	AeCh
45	4	0.11	40.1	87.34	64.75	5.6	1.54	18.70	7.57	0.61	0.52	84.23	PrIn
46	4	0.12	26.7	79.02	73.41	5.9	0.95	9.70	8.99	1.43	0.13	350.00	No
47	4	0.12	11.5	74.19	75.35	6.8	0.26	31.10	1.83	3.09	0.38	49.18	AeCh
48	4	0.13	31.8	77.20	84.05	7.3	0.29	0.00	0.31	1.94	0.5	87.80	No
49	4	0.13	19.7	83.48	86	7	0.64	11.80	2.41	1.32	0.35	75.49	AeCh
50	4	0.14	21.6	86.63	57.64	7.3	0.39	23.70	2.94	1.10	0.34	117.06	AeCh, PrIn
51	4	0.15	56	85.88	65.35	5.7	1.38	5.80	15.88	1.30	0.36	113.64	AeCh
52	4	0.15	21.3	78.29	73.15	6.6	0.75	10.70	6.83	3.03	0.48	93.71	AeCh
53	4	0.16	15	84.14	38.76	5.3	1.02	30.30	10.73	1.21	0.25	168.88	No
54	4	0.18	21.3	86.52	77.02	7	0.48	7.30	6.04	2.32	0.38	110.58	AeCh
55	4	0.19	26.1	85.09	69.2	6.2	1.67	27.10	11.31	1.63	0.26	171.85	Unknown

Table F-1 (Continued)

Log No.	DC	Dn	Di	WA	WC	pH	P	NDS	SS	F	N	C/N	Occurrence/ Species
56	4	0.21	12.4	75.28	75.72	6.9	1.59	19.30	4.36	3.74	0.55	35.73	AeCh, DoTi, PrBu
57	4	0.21	15.9	69.83	72.65	5.4	1.98	22.20	8.94	2.72	0.62	73.71	No
58	4	0.22	15	69.75	68.17	6.7	0.39	13.20	3.28	2.58	0.34	82.85	AeCh
59	4	0.24	11.5	64.17	63.53	5.8	2.04	16.20	9.09	3.45	0.84	48.07	AeCh, PrIn
60	4	0.26	32.5	63.96	52.94	6.5	2.23	6.10	6.94	0.63	0.22	212.73	No
61	4	0.28	26.1	45.02	38.49	7.1	15.39	61.10	32.46	1.47	0.21	230.05	OdSi
62	4	0.30	11.1	67.60	61.68	6.3	0.73	23.90	12.20	4.08	0.7	42.56	PrJe
63	4	0.36	14.6	72.82	67.11	7.2	0.72	14.90	5.31	1.98	0.25	32.00	PrJe
64	5	0.08	26.7	87.68	69.16	6.5	0.73	13.40	2.73	3.13	0.55	78.18	No
65	5	0.09	36.3	68.20	85.44	7.2	0.63	17.80	3.46	2.11	0.33	126.12	No
66	5	0.10	44.6	61.28	69.74	6.9	0.12	18.60	1.36	2.45	0.41	93.34	AeCh
67	5	0.10	22.9	88.33	79.61	7.1	0.50	33.20	3.52	3.37	0.89	43.55	AeCh
68	5	0.11	21	76.08	84.98	7.4	0.75	12.80	4.31	2.73	0.38	105.92	AeCh
69	5	0.13	26.1	85.09	67.94	6	2.91	11.70	10.20	6.88	0.71	41.62	No
70	5	0.13	11.8	58.84	4.25	7	0.65	15.00	5.04	1.52	0.43	95.67	NiSp1

Table F-1 (Continued)

Log No.	DC	Dn	Di	WA	WC	pH	P	NDS	SS	F	N	C/N	Occurrence/ Species
71	5	0.13	15.9	74.71	65.44 5	6.6	1.33	19.68	5.51	2.92	0.47	87.53	PrBu
72	5	0.15	22.9	81.73	73.61	5.6	1.71	18.80	8.25	2.22	0.41	110.73	No
73	5	0.18	24.2	72.19	60.84	6.5	0.23	9.90	2.57	3.53	0.27	31.07	No
74	5	0.23	22.9	67.64	58.88	5.6	5.06	45.60	13.67	1.24	0.32	149.06	No
75	6	0.10	20.7	63.75	32.26	7	1.06	27.50	4.15	2.35	0.64	62.89	NiSp2
76	6	0.17	44.6	77.49	66.22	5.5	1.22	27.80	5.62	1.40	0.27	163.59	No
77	6	0.19	13.4	76.72	77.48	7	0.00	0.00	0.00	1.33	0.38	119.89	No
78	6	0.35	42.3	59.39	52.31	6.5	1.64	6.60	0.99	2.43	0.36	54.47	No
79	1+2		49.7										No
80	1+2		21.6										No
81	1+2		15.3										No
82	1+2		18.5										No
83	1+2		20.4										No
84	1+2		31.2										No
85	1+2		15.3										No

Table F-1 (Continued)

Log No.	DC	Dn	Di	WA	WC	pH	P	NDS	SS	F	N	C/N	Occurrence/ Species
86	1+2		35										No
87	1+3		23.2										No
88	1+3		55.4										No
89	1+3		23.6										No
90	2		45.2										No
91	2		38										No
92	2		35										No
93	2		28.6										No
94	2		19.7										No
95	2		21.6										No
96	2		13.4										No
97	2		26.1										No
98	2		31.2										No
99	2		38.2										No
100	2		14										No

Table F-1 (Continued)

Log No.	DC	Dn	Di	WA	WC	pH	P	NDS	SS	F	N	C/N	Occurrence/ Species
101	2		26.7										No
102	2		19.1										No
103	2		16.5										No
104	2		29										No
105	2		19.1										No
106	2		24.2										No
107	2		13.0										No
108	2		15.3										No
109	2		38.2										No
110	2		26.4										No
111	2		15.3										No
112	2		31.2										No
113	2		14.6										No
114	2		28										No
115	2		22.6										No

Table F-1 (Continued)

Log No.	DC	Dn	Di	WA	WC	pH	P	NDS	SS	F	N	C/N	Occurrence/ Species
116	3		42.3										No
117	3		21										No
118	3		35.7										No
119	3		44.6										No
120	3		25.5										No
121	3		14.3										No
122	3		18.1										No
123	3		28.6										No
124	3		21.3										No
125	3		13.4										No
126	3		11.5										No
127	3		31.2										No
128	3		17.2										No
129	3		31.8										No
130	3		28										No

Table F-1 (Continued)

Log No.	DC	Dn	Di	WA	WC	pH	P	NDS	SS	F	N	C/N	Occurrence/ Species
131	3		21										No
132	3		10.2										No
133	3		14										No
134	3		14.3										No
135	3		17.2										No
136	3		11.1										No
137	3		13.7										No
138	3		13.4										No
139	3		15.9										No
140	3		10.2										No
141	3		19.7										No
142	3		25.5										No
143	3		14.6										No
144	3		16.5										No
145	3		20.7										No

Table F-1 (Continued)

Log No.	DC	Dn	Di	WA	WC	pH	P	NDS	SS	F	N	C/N	Occurrence/ Species
146	3		24.2										No
147	3		18.5										No
148	3		12.4										No
149	3		26.1										No
150	3		35.7										No
151	3		25.8										No
152	3		14.3										No
153	3		26.4										No
154	3		17.8										No
155	3		38.8										No
156	3		26.7										No
157	3		45.8										No
158	3		24.8										No
159	3		24.2										No
160	3		47.7										No

Table F-1 (Continued)

Log No.	DC	Dn	Di	WA	WC	pH	P	NDS	SS	F	N	C/N	Occurrence/ Species
161	3		22.0										No
162	3		27.7										No
163	3		29.3										No
164	3		21.6										No
165	3		21.6										No
166	3		18.5										No
167	3		11.5										No
168	3		29.9										No
169	3		28										No
170	3		37.6										No
171	3		16.6										No
172	3		15.3										No
173	3		21										No
174	3		11.1										No
175	3		20.7										No

Table F-1 (Continued)

Log No.	DC	Dn	Di	WA	WC	pH	P	NDS	SS	F	N	C/N	Occurrence/ Species
176	3		25.5										No
177	3		27										No
178	3		27										No
179	3		21										No
180	4		15.9										No
181	4		16.9										No
182	4		43.3										No
183	4		17.2										No
184	4		13.1										No
185	4		13.7										No
186	4		11.5										No
187	4		24.8										No
188	4		17.2										No
189	4		19.7										No
190	4		26.7										No

Table F-1 (Continued)

Log No.	DC	Dn	Di	WA	WC	pH	P	NDS	SS	F	N	C/N	Occurrence/ Species
191	4		12.7										No
192	4		17.5										No
193	4		33.1										No
194	4		11.4										No
195	4		60.5										No
196	4		37.6										No
197	4		20.7										No
198	4		21.6										No
199	4		20.1										No
200	4		28.0										No
201	4		21										No
202	4		47.7										No
203	4		33.1										No
204	4		12.1										No
205	4		22.6										No

Table F-1 (Continued)

Log No.	DC	Dn	Di	WA	WC	pH	P	NDS	SS	F	N	C/N	Occurrence/ Species
206	4		33.7										No
207	4		28.6										No
208	4		19.1										No
209	4		49.7										No
210	4		10.2										No
211	4		17.8										No
212	4		24.2										No
213	4		14.9										No
214	4		14										No
215	4		15.3										No
216	4		12.1										No
217	4		28										No
218	4		12.7										No
219	5		25.5										No
220	5		47.7										No

Table F-1 (Continued)

Log No.	DC	Dn	Di	WA	WC	pH	P	NDS	SS	F	N	C/N	Occurrence/ Species
221	5		15.3										No
222	5		14										No
223	5		12.1										No
224	5		23.6										No
225	5		13.4										No
226	5		19.7										No
227	5		33.7										No
228	5		12.7										No
229	5		24.2										No
230	5		13.7										No
231	5		29.9										No
232	5		14.0										No
233	5		18.5										No
234	5		18.5										No
235	5		22.9										No

Table F-1 (Continued)

Log No.	DC	Dn	Di	WA	WC	pH	P	NDS	SS	F	N	C/N	Occurrence/ Species
236	5		20.4										No
237	5		15.3										No
238	5		15.6										No
239	5		12.1										No
240	5		10.8										No
241	5		15.9										No
242	5		16.5										No
243	6		12.1										No
244	6		14.0										No
245	6		18.4										No
246	6		14.6										No
247	6		15.9										No
248	6		12.7										No
249	6		10.8										No
250	6		12.4										No

Table F-1 (Continued)

Log No.	DC	Dn	Di	WA	WC	pH	P	NDS	SS	F	N	C/N	Occurrence/ Species
251	6		23.9										No
252	6		14.6										No
253	6		12.1										No
254	6		15.0										No
255	6		10.2										No
256	6		20.7										No
257	6		13.4										No
258	6		34.4										No
259	6		14.0										No
260	6		31.8										No
261	6		15.6										No
262	6		20.4										No
263	6		29.3										No
264	6		18.5										No
265	6		13.4										No

Table F-1 (Continued)

Log No.	DC	Dn	Di	WA	WC	pH	P	NDS	SS	F	N	C/N	Occurrence/ Species
266	6		14.3										No
267	6		11.8										No
268	6		21.6										No
269	6		11.5										No
270	6		13										No

Table F-2 Properties of logs and wood collected from Bangkok metropolitan area, Thailand

Log No.	DC	Dn	Di	WA	WC	pH	P	NDS	SS	F	N	C/N	Occurrence/ Species
1	2	0.24	12.4	69.66	63.19	7.4	0.23	17.85	120.40	6.90	0.88	50.37	AeCh
2	2	0.29	9.5	67.53	69.28	6.8	0.43	16.44	48.48	3.73	0.86	48.55	AeCh
3	2	0.26	6.4	64.15	70.86	7.1	0.71	16.14	113.00	6.56	0.88	52.15	AeCh
4	2	0.30	11.4	68.34	61.33	7.2	0.55	22.57	130.21	11.15	1.46	31.64	AeCh
5	3	0.21	10.5	69.87	73.43	7.2	0.22	17.87	29.05	10.16	1.54	28.25	AeCh
6[a]	-	0.21	-	92.01	78.72	7.5	0.15	7.62	26.36	3.98	0.47	94.66	AeCh

[a] Log No.6 is sawdust in plastic bag.

Wood properties: DC = Decay class; Dn = wood density (g/cm^3); Di = diameter (cm); WA = water absorption capacity; WC = water content (%); P = total phenol (mg/g); NDS = neutral detergent fibre (%); SS = total soluble sugar (mg/g); F = fungal biomass (mg/g); N = nitrogen content (%); C_N = carton to nitrogen ratio

Stag beetle species: AeCh = *Aegus chelifer chelifer*

APPENDIX G

CLIMATE DATA OF CHANTHABURI (WEATHER STATION 480201) DURING 1995–2014

Table G-1 Total rainfall (mm) at weather station 480201-Chanthaburi

	Month												
Year	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Average
1995	20.3	48.7	53.4	68.3	385	705.4	529.9	736.9	699.4	350.2	10.7	1.9	300.8
1996	5.1	59.1	14.7	122.6	372.6	378	417.3	325.5	512	303.2	120.7	T	219.2
1997	6.6	90.4	150	78.1	204.9	165.9	512	378.9	436.2	257.8	42.5	0	193.6
1998	0	22	3.4	21.4	499.8	867.7	520.3	443.4	569.9	168.6	18.2	17	262.6
1999	1.1	25.4	55.8	463.4	480.4	394	731.4	331.7	619.9	308.5	97.8	0.3	292.5
2000	35	28	25.5	179.8	382.1	720.3	516.7	488.8	325.1	235.5	74.9	18.8	252.5
2001	70.9	12.2	208.5	180.2	495.5	254.5	337.7	301.5	360.6	276.4	27.5	3.1	210.7
2002	1.1	2.7	36.6	77.9	556.4	504.7	317.5	445.7	376.5	237.3	40.2	52.4	220.8
2003	0	55.4	40	91.1	129.2	462.2	599.2	580.7	377.2	137	0.9	0	206.1
2004	157.6	17.8	44.6	74.5	389.8	489.5	904.3	512.7	233.2	137.6	27.2	0	249.1
2005	23.5	0.6	50.3	171.7	330.9	623.7	362.9	442.8	457.9	239.9	179.5	7.4	240.9
2006	0	59.4	60.1	101.7	466.2	509	585.1	626.3	581	829.7	90.7	1.3	325.9
2007	19.1	26.4	161.9	272.1	578.4	548.2	851.9	299.8	519.9	144.1	13.1	0	286.2
2008	43	91.5	55.5	164.5	766.2	458.8	514.8	357.1	695.9	231.1	74	0	287.7
2009	0.5	0.1	243.2	186.2	633.3	258.1	543.4	236.7	689.4	307.7	0.5	0	258.3
2010	30.2	100.2	77	159.1	281.6	492.8	567.4	512.7	225.9	375.3	5.7	28.2	238
2011	0	32.9	100.3	193.9	290.9	534.4	422.2	563	860.7	264.2	54.2	0	276.4
2012	61.2	101.8	91.4	60.9	545.9	286.6	475.1	245.5	379.5	172.6	204.4	0	218.7
2013	66.8	43.7	61.2	223.8	140.8	562.3	1035.4	498.7	46.9	327	72.1	2.7	256.8
2014	0	40.2	115.8	55.3	170.6	548.3	496	276	718	259.5	76.7	12.3	251.7

Table G-2 Rainy days at weather station 480201-Chanthaburi

	Month												
Year	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Average
1995	2	5	5	13	15	27	23	29	29	21	4	1	14.5
1996	1	3	3	8	24	18	24	23	26	17	9	0	13
1997	1	5	4	10	14	16	24	18	18	16	8	0	11.2
1998	0	5	4	5	18	28	25	21	28	18	8	3	13.6
1999	2	4	6	21	28	20	23	23	27	18	9	1	15.2
2000	5	7	8	21	23	23	28	23	19	22	5	2	15.5
2001	6	2	20	10	25	22	22	27	25	25	5	2	15.9
2002	1	3	9	12	26	25	21	28	25	12	6	7	14.6
2003	0	5	10	5	21	27	27	23	26	16	3	0	13.6
2004	5	5	3	8	23	21	27	24	23	9	1	0	12.4
2005	2	1	4	8	22	30	26	20	29	17	11	5	14.6
2006	1	6	6	16	20	28	23	26	27	22	8	1	15.3
2007	2	4	8	17	29	26	29	22	26	18	5	0	15.5
2008	2	6	9	15	30	23	28	26	27	20	11	0	16.4
2009	1	3	16	18	26	22	24	22	25	22	1	0	15
2010	6	6	5	8	18	27	27	30	22	22	5	3	14.9
2011	1	8	12	12	23	28	27	26	24	23	5	2	15.9
2012	13	8	7	11	28	26	27	21	29	19	15	0	17
2013	7	4	5	12	20	26	26	24	1	19	8	2	12.8
2014	-	8	5	6	16	26	24	18	25	20	10	1	14.5

Table G-3 Average relative humidity (%) at weather station 480201-Chanthaburi

	Month												
Year	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Average
1995	67	72	78	78	82	84	86	87	89	83	72	65	79
1996	70	66	76	79	84	84	86	85	88	83	79	67	79
1997	72	79	82	80	81	82	85	84	86	84	76	68	80
1998	75	79	75	77	82	87	85	85	88	81	74	69	80
1999	67	72	79	85	87	85	86	85	86	83	78	64	80
2000	72	72	77	82	83	85	86	84	84	86	69	68	79
2001	75	75	81	80	84	83	84	84	86	86	71	68	80
2002	71	77	77	79	86	84	82	87	86	79	74	73	80
2003	64	74	78	76	82	84	86	85	86	81	70	63	77
2004	73	73	76	77	82	84	85	85	85	75	68	63	77
2005	73	80	74	78	83	85	85	83	86	81	77	69	79
2006	69	73	76	78	81	84	84	85	85	82	72	63	78
2007	62	73	79	81	86	84	84	84	85	79	65	67	77
2008	66	71	74	81	86	84	84	82	86	82	72	62	77
2009	62	75	78	79	84	80	83	81	86	85	70	69	78
2010	73	79	74	77	79	85	85	87	83	83	68	68	78
2011	57	76	74	81	81	85	83	84	87	82	69	61	77
2012	74	78	76	78	84	81	85	82	88	82	82	69	80
2013	69	75	75	79	81	86	88	85	87	84	76	66	79
2014	62	79	78	77	80	84	82	83	86	82	75	63	78

Table G-4 Average temperature (°C) at weather station 480201-Chanthaburi

	Month												
Year	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Average
1995	26.3	26.6	28	29	28.5	28.1	27.3	27.5	26.7	27.1	26.9	25.3	27.3
1996	26.2	26.7	28.1	28.8	28.2	27.9	27.2	27.7	27	27.1	26.8	25.1	27.2
1997	25.4	27.2	27.7	27.9	28.7	28.6	27.7	28	27.4	27.4	27.6	27.7	27.6
1998	28.1	28.6	29.3	29.8	29.2	28.3	28.1	27.9	26.9	27.5	26.9	26	28.1
1999	26.9	27.4	28.5	27.8	27.1	27.4	27.5	27.3	27.2	26.9	26.5	23.6	27
2000	26.8	26.7	28	28	28.4	27.4	27.3	27.6	27.3	26.8	26.3	27.3	27.3
2001	27.2	27.4	27.7	29	28.2	28	28.1	27.8	27.6	27.1	25.5	26.3	27.5
2002	26.4	27.4	28.5	28.6	27.7	28.4	28.6	27	27.3	27.6	27.8	28	27.8
2003	26.7	27.6	28.1	29.3	28.7	28	27.4	27.9	27.2	27.3	28	25.8	27.7
2004	26.6	26.7	28.6	29.2	28.7	27.7	27.6	27.8	27.3	27.9	28.2	26.2	27.7
2005	26	28	28	29	28.9	28.6	27.9	28.1	27.4	27.6	27.1	25.5	27.7
2006	26.8	28.1	28.6	28.8	28.2	27.9	28	27.4	27.2	27.4	28.1	26.5	27.7
2007	26.9	26.9	28.5	28.2	27.9	28.5	27.5	27.9	27.5	27.4	26.2	27.3	27.6
2008	26.9	27	28.2	28.2	27.7	28.1	27.5	27.9	27.1	27.7	26.4	25.9	27.4
2009	25.2	27.8	28	28.8	27.9	28.9	27.8	28.3	27.3	27.1	27.1	27.1	27.6
2010	27.2	28.4	28.8	29.8	29.7	28.5	28	27.3	28.1	27.1	27.5	27	28.1
2011	26.8	27.3	26.9	28	28.9	28	28	27.7	27.2	27.5	28	26	27.5
2012	27.3	28	28.8	29	28.2	28.7	27.7	28.2	27	27.6	28	28.1	28
2013	27.1	28.3	29	29.2	29.4	28.1	27.3	27.8	27.3	27.2	27.6	24.5	27.7
2014	25	26.3	28.5	29.3	29.4	28.8	28.5	28	27.5	27.5	28.2	26.9	27.8

VITA

Mr. Nut Songvorawit was born on August 18th, 1986 in Bangkok, Thailand. He received the B.S. (Biology) with First Class Honours in 2009 and the M.S. (Microbiology) in 2011 from the Department of Microbiology, Faculty of Science, Kasetsart University. He continued his study for Doctoral degree at the Department of Biology, Faculty of Science, Chulalongkorn University and received the Ph.D. in Zoology in 2017. During his education, he has received academic awards as follows:

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- Certificate of outstanding academic performance for Master student, academic year 2010

- Professor Dr. Tab Nilanidhi Foundation Award for academic excellence in the Master programme in Microbiology, Kasetsart University, 2011

- Second runner-up in oral presentation award of the 20th Biological Science Graduate Congress (BSGC) at Chulalongkorn University, 2015