

Studies on mass culture and aggregation pheromones in
the exotic powderpost beetle, *Lyctus africanus* Lesne
(Coleoptera: Lyctinae)

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2015

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I. General introduction

Pest distribution has long been associated with the movement of people and materials due to rapid increases in economic development among countries. Several alien species of the wood pest Lyctinae subfamily, belonging to the Bostrichidae family [1], have been introduced in countries such as Australia [2-4], New Zealand [5,6], USA/Canada, Europe, countries around the Mediterranean [7-10] and Japan [9,11,12]. Species of the subfamily Lyctinae are reported to be distributed in all the faunal regions, but are supposed to be an endemic species of oriental and Ethiopian regions [7].

Nowadays, Japan is importing an enormous number of wood products due to the sustained international trade in Japanese economy. It is thought that many alien species have been unintentionally introduced into Japan via seaports and airports along with imported goods and containers, and by staff members [11]. Due to this mass introduction, some of the alien species have established habitations in Japan. It was reported that some Lyctinae, *Lyctus africanus* (Lesne), *Lyctus brunneus* (Stephens), *Minthea rugicollis* (Walker), *Lyctus linearis* (Goeze), and *Lyctoxylon dentatum* (Pascoe) had established in some prefectures in Japan and being registered as alien species [11,12]. Furthermore, *L. africanus* was discovered frequently in this country rather than the other species [12-14]. Nowadays, the cases of *L. africanus* are increasing in Japan.

In Australia, *L. africanus* is considered as high in entry, establishment, and spread potential as well as its economic and environmental impact [2]. In addition, five introduced species, belonging to sub family Lyctinae, are

widespread in the whole country. *L. africanus* has also been recorded in modern industrial countries like North America, and European countries [9].

Recently, *L. africanus* has become an important pest owing to its migration into new countries, and it has been considered to be one of the major pests threatening timber and wood products, including plywood [7,9] [14,9], dried roots, seeds and tubers [7,15]. This beetle attacks sawn hardwood timber products and also damage many manufactured wood products. It may also infest dried roots and tubers [15]. Unfortunately, extensive study on *L. africanus* is not available rather than the other species, *L. brunneus*.

The Lyctines are popular with the term of 'true powderpost beetles' with regard to excavating the larvae tunnel, feeding on wood, and converting it into fine powder. Lyctine damage is inconspicuous, and, infestations are usually discovered belatedly due to the difficulty of locating and monitoring them. Thus, further information on relationship between Lyctine and the environment are needed.

The Lyctines are present throughout the year in buildings but are most commonly found during warmer months due to their increased activity. The most obvious and often sign of infestation is the presence of exit holes. External signs of damage are not readily apparent until the adults emerging.

However, the presence of emergence holes does not necessarily signify an active infestation. Lyctine damage is mostly identified belatedly by reason of poor knowledge and skill to locate and monitor the infestation. A previous research reported that generally three variables govern susceptibility to Lyctine attack in sapwood: vessel sizes, nutrition and moisture content [3].

To determine the Lyctine population in its natural habitat, it is necessary

to examine the degree of infestation. Some monitoring techniques have been developed to regularly inspect insect populations. The use of insect traps for monitoring has been widely used in pest management against stored-product pests [16]. Traps enable insects to be detected at very early stages of infestation (early warning), and improve the chances of preventing damage. Generally, insect traps are combinations of attractants and killing systems. Many kinds of attractants are widely used, including food baits, pheromones, light and colored surfaces [17]. Unfortunately, there is still no device for detecting and monitoring Lyctine beetles in the field due to the lack of sufficient data on their ecological features. Thus, strategies for monitoring and controlling this beetle by examining the Lyctine ecology are urgently needed.

In laboratory, *L. africanus* was frequently found showing a behavior of forming a group. This behavior was exhibited within a few minutes after releasing into a Petri dish. The phenomena of forming a group by many individuals is known as an aggregation [18]. Generally, the aggregation behavior is induced by some factors, such as specific chemical compound or pheromone which initiate individuals forming a group. The activity of aggregation behavior is a sort of communication in insect. Intra- and interspecific communications are absolutely essential for survival and reproduction of insects.

To accomplish the study, establishment of the mass culture of *L. africanus* was also elaborated. The development of mass culture of *L. africanus* is urgently needed to supply large number of beetles to conduct comprehensive study on *L. africanus*.

First of all, in Part I, mass culture of *L. africanus* in laboratory is discussed. The study is emphasized on finding the standard diet for rearing *L. africanus* in laboratory. Study on Lyctines in laboratory scale has been practically done in an artificial diet instead of wood block. Researchers have developed several methods for Lyctines by using wood- and non-wood-based diets [15,19,20]. However, limited results on mass culturing of *L. africanus* with artificial diets have been reported.

This study aims at the establishment of the mass culture and monitoring system for *L. africanus*. On this study the diet composition for rearing the *L. africanus* in laboratory was evaluated. By understanding the favorable diet composition in *L. africanus*, it must become possible to supply more *Lyctus* adult in laboratory for further study on aggregation behavior of *L. africanus*.

As mentioned earlier, aggregation behavior is a sort of communication in insect. A communication is considered if one individual gives off any signal that produces a change in the behavior of another individual [21]. The signals can be observed as visual, tactile, acoustic, and chemical as being the primary means of communication in insect [21]. In general, the aggregation behavior is typically activated by chemical signal. Chemical communication of *L. africanus* is initially examined in order to gain further understanding of their behavior in a group. By understanding the communication among beetles, further inspection and controlling of beetles population are possible to develop the monitoring technique. In Part II, the behavior was investigated using a chemical approach through comprehensive screening of the potential compounds produced by *L. africanus*. This study will contribute significantly to the understanding of the *Lyctus* ecology for the purpose of developing

monitoring techniques for *Lyctus* beetles.

II. Biology of *Lyctus africanus*

Lyctus africanus is a member of subfamily Lyctinae in the beetle family Bostrichidae [1]. The subfamily Lyctinae consists of three tribes: Lyctini, Trogoxylini and Cephalotomini [1], and *L. africanus* beetle belongs to tribe Lyctini.

The subfamily Lyctinae is characterized as small (2.5 – 4 mm of body length), reddish-brown to black beetles, with a flattened dorso-ventrally head and lateral eyes [7]. The mandibles are heavily chitinized with outer margin often bears a patch or brush of thick setae. The antennae are claviform, eleven-segmented, which are immediately anterior of the eyes. The legs are slender with simple tarsal claw. Elytra have uniform shape, covered with irregularly placed punctations and hairs. The abdomen side composes of five visible sternites.

Sex characteristics between male and female of Lyctinae could be identified by the presence of external characters [22]. In general, the size difference between male and female Lyctinae could not be guided to sex characteristic. The female is recognized by the existence of heavy fringe of silky hairs on distal border of the fourth sternite and median tuft on the fifth sternite [7].

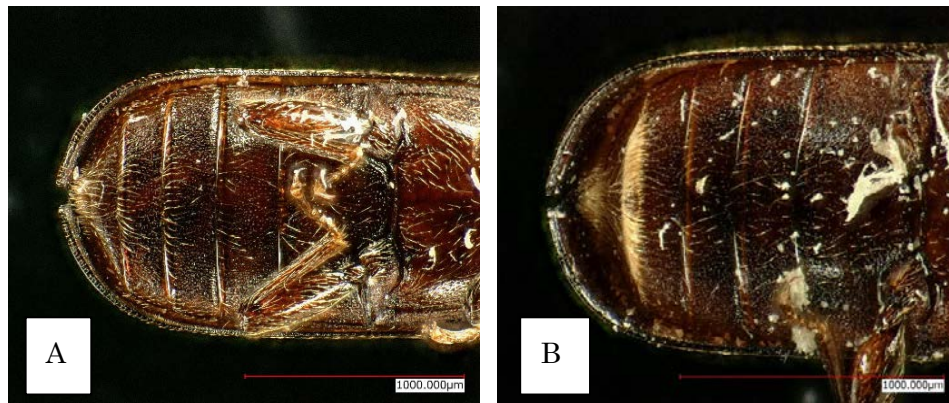


Fig. II.1. Sexual characteristic of adult *L. africanus* ; male (A) and female (B) beetles.

Copulation occurs shortly after emergence because the new emerged beetles are sexually mature upon emergence [23]. Premating period of newly emerged *L. africanus* lasts 48 hours after emergency [24]. Then, copulation is observed either at dusk or during at night which indicated by mating behavior by a pair of female and male beetle. In *L. africanus*, the male chase and ride over the female, then coitus continues for 2 to 10 minutes throughout the mating process [24]. Copulation may occur more than once, considering from observations on living and dissected beetles [22]. In general, one male fertilize several females considering the excess number of females than males [23].

The female may taste the wood surfaces by gnawing the torn fibers, generally in transversally, to detect wood containing quantities of food sufficient for normal development [25,24], then resulted the tasting mark on wood surfaces. The making of tasting marks is closely related with oviposition, which is important to open the wood vessels to insert the ovipositor [25]. The ovipositor is a long, slender tube, and flexible with a chitinized inner wall and a colorless transparent outer sheath [22]. During oviposition, the female extends the ovipositor from the body to insert directly into the lumen of the

vessel, tracheae, or pores of the wood [7]. The length of ovipositor may exceed the body when everting in the form of single tube [22].

The oviposition takes place at dusk or during the night after two to four days of *L. africanus* emergence [24], and then the eggs are laid longitudinally in a single or in group within the trachea, or the wood vessel, and or the open pore of the hardwood by the fertilized females [23]. The chosen pores are slightly greater than the normal diameter of the egg [22]. The number of laid eggs depends on the size, suitability and capacity of the vessel or the pore.

Generally, number of laid eggs in a single pore varies from 1 to 8, however it was also varies according to the *Lyctus* species [22]. It had been reported that *L. africanus* laid 32 (4 to 72) eggs, on average under room condition [24]. In the case of unfertilized females, they lay nothing because the ripe eggs become swollen in the calices before leading to the death of females [23].

The egg is translucent, white, and cylindrical which is tapered and rounded at the posterior pole. The anterior pole is rounded with a long, slender tube-like process terminated in a round protuberance [23]. The size of eggs is 650 μm in length and 120 μm in diameter [26], and it slightly increases in size during maturation [23]. The development of the eggs starts at the fourth day after oviposition, which is indicated by the color changing of the posterior half of the egg to be a little more transparent than the anterior half of the egg [26]. Then, this development is followed by the appearance of the embryo's head near the center of the egg, and the embryo subsequently consumes the residual egg yolk mass in next three to five days [26]. The embryo continuously moves forward, filling the posterior end. The anterior portion is occupied by a mass which consists of large yolk granules and fat

bodies, then the young larva emerges through the anterior end of the shell [26]. Thus, the eggs hatch 7-15 days after oviposition [23,25,26].

The young larvae start tunneling through the wood in the direction of the grain, reducing the wood to a fine powder which is packed in the galleries. They feed and grow until reaching maturity. The fully grown larva approaches the surface of the wood, constructs the pupal chamber just below the wood surface and then pupates [22]. The pupation lasts for 3-4 weeks. The *Lyctus* molt the pupal skin, remains within the chamber for another 3-4 days to harden the chitin before eating its way to the outside through the exit hole (2 mm in diam). Then followed by emergence phase and recommence the cycle [25]. The newly emerged *Lyctus* are ready to mate [23,7]. After mating, the females start egg-laying, thus completing the life cycle. One generation is obtained once a year in the field, though in laboratory conditions beetles can be obtained all the year ground.

The length of life span of both female and male was three to six weeks [23,25]. On the whole, there is little difference in length of survival between the two sexes. Through this period, copulation and oviposition occurred. The longevity of both male and female *L. africanus* was reported being prolonged by mating rather than that of by un-mating beetles [24].

The activity and reactions of the beetles are closely related to the conditions of temperature, light and humidity. The beetles become very active at temperatures above 20°C. In this condition, the beetles readily take flight toward the light source (positively phototropic) and are quite a strong fliers [22]. Throughout the day, the beetles crawl into the small spaces, such as old exit hole and any cracks, but become active at dusk.

The Lyctinae is commonly known as powderpost beetle regarding the activity of its larval stage to convert sapwood, particularly hardwood, into powdery or pelleted frass [7]. The beetles are frequently found in dried and cured lumber of hardwood. They are more often found in rather recently dried wood than in old wood. Powderpost beetles may infest the hardwood flooring, hardwood timbers, plywood, and wood articles such as crating and furniture [27]. Several generations may re-infest the same wood piece until it is riddled with exit holes and only the outer shells remain. Once the wood is infected for the first time, external evidence of such infestation is obscure even though the larvae may cause extensive internal damage. The infestations are detected by the presence of powdery frass around infested wood, as the larvae tunnel their way out through the sapwood, and exit or flight holes (2-3 mm in diam) are detected on the surface of the wood [7]. By cutting the infested wood, galleries packed with powdery frass are noticeable.

Generally, the beetles undergo the inactive periods within the wood during late October to early April, then emerge from the infested wood mainly during the warm seasons; June, July, and August.

Some factors influencing attack by *Lyctus* have been investigated. There is relation between the diameter of the vessels of a wood and its liability to attack [22]. *Lyctus* may lay their eggs in the pores or vessels, thus the preferred wood should have optimum not too large nor small pore diameter than the egg diameter. Hence, the small-pored timber should not be liable to infestation as oviposition cannot take place. The starch and moisture contents of the wood also reported to govern the susceptible of *L. africanus* infestation on woods [24]. It is reported that a wood with a moisture content of less than

8% is not attacked by *Lyctus* beetle [7].

As mention earlier, some Lyctinae had established in some prefectures in Japan, such as *L. africanus* Lesne, *L. brunneus* Stephens, *Minthea rugicollis* Walker, *L. linearis* Goeze, and *Lyctoxylon dentatum* Pascoe. All of them are approximately identical with few differences. The *L. africanus* is similar with *L. brunneus*, however they can be distinguished by some features. Generally, *L. africanus* is smaller, on the average, than *L. brunneus*. In addition, the female of *L. africanus* can be distinguished by the heavy fringe of hairs on the distal margin of the fourth sternite, which is lacking in *L. brunneus* [7].

III. Insect communication

Communication is considered between two or more individuals if one individual transmits any signals that produces a change in the behavior of another individuals which increase fitness of the participants [21]. Various signals used in insect communication are identified as visual, acoustical, tactile, and chemical as being the primary means of communication. Basically, communication is useful in survival and reproduction of insect. The communication itself is classified according to the receptors involved in receiving stimuli. The followings are some differences of communication used by insect.

1. Visual communication

Visual signal is primarily important to identify food and mates, and also to orient themselves in the environment. The receptor of visual signal is the compound eyes which have the best resolution of the optical receptors [21].

2. Acoustic communication

It is kind of communication using vibrational signals produced by insect to the air, water, or solid substrates, then received and interpreted by other insects. Vibrations may be produced by a variety of mechanisms, such as percussion, vibration, substrate vibration, and air expulsion, and through variety of substrates to ultimately reach another insect and change its behavior [21].

3. Tactile communication

Generally, the tactile signals are used for short-range communication, which is useful for aggressive or sexual encounters. Generally, many insects must touch their mates before copulation, then receiving close-range chemical signals that release the required stepwise behaviors [21].

4. Chemical communication

Insect uses chemical cues governing of their behavior and most of their interactions. The chemicals that are released into the surrounding medium are valuable for communicating to find a mate, gather together, provide others of their own species with the location of food, identify nest mates, and defend themselves against predators [21]. Chemical used in communication is classified based on the functional roles of those chemicals in their interactions. There are two types of chemical communication; hormone and semiochemicals [21]. The hormone is a chemical produced by an organism and mediate physiological reactions within that organism, while the semiochemical is any chemicals produced that mediates an interaction between two organisms whether of the same or different species. In semiochemicals, the chemicals used in mediating interaction between

members of the same species (intraspecific interactions) is categorized as pheromones, while the chemicals mediate the interaction between members of different species (interspecific interactions) is considered as allelochemicals.

Pheromones are chemicals produced by specialized glands (exocrine glands) that are secreted to the outside by one animal and have a specific effect on another individual of the same species [21]. Most pheromones consisted of multicomponent blends of compounds in different concentration, rather than a single signal. According to the way in which of pheromones influence other physiological systems, pheromones are divided into releaser and primer pheromones. A primer pheromone stimulates a fundamental physiological change in the receiver, causing another behavior to be expressed. On the other hand, a releaser pheromone acts on the central nervous system and cause the immediate release of behavior.

The most common functional category of releaser pheromones include sex pheromones, aggregation pheromones, alarm pheromones, trail pheromones, and epideictic or spacing pheromones. A sex pheromone is known as chemical released by one or both sexes; generally benefit both emitter and receiver. In some cases, the pheromones also may be used to assess the quality of the individual as a mate [28]. Unlike sex pheromones, the aggregation pheromones induce aggregations of conspecifics by bringing many conspecifics of both sexes together [28]. The potential benefits of aggregation pheromones are to improve the exploitation of a resource and/or increases the probability of locating a mate, and also to

decrease the risk of predation or parasitism of all group members. In particular conditions, alarm pheromone is released by most social insect in response to danger, such as an attack by a predator which benefits the receivers by modifying their behavior to defend the colony or to reduce the probability of being captured. Another type of pheromone produced by most social insect are trail pheromone. The pheromone is helpful to indicate the location of exploitable resources with respect to the position of the colony, and benefits the colony by increasing the efficiency of foraging [28]. Different type of pheromone is also produced by insect to indicate that a resource has already been exploited in order to maintain the density of individuals attempting to exploit an exhaustible resource, usually by marking an oviposition site after egg-laying as a warning to other females [21]. The pheromone is deposited on the surface or inside a resource, and give benefits the marker by reducing competition for its progeny [28].

The pheromone productions may occur throughout the insect's adult life, however the pheromones are generally released only under certain environmental and physiological circumstances [21]. Females that mate more than once release pheromones periodically, but those females that only mate once usually terminate their pheromone release after mating. The pheromones are generally produced by modified epidermal cells that can be found in various places throughout the body. These are often clustered into groups designated exocrine glands, which are secretory glands that direct their products to the outside of the organism [21].

In this study, intraspecific interactions within members of *L. africanus* beetles were evaluated. Potential chemical cues within this

species were evaluated and detected in order to gain more understanding of individuals' interactions.

IV. Part I. Mass culture of the exotic powderpost beetle, *Lyctus africanus* Lesne

Chapter 1. Fecundity of powderpost beetle, *Lyctus africanus*, on different diets.

1.1. Introduction

First initiation of infestation is to find the suitable host is commonly affected by nutritional quality of the host. Commonly, host-finding is initiated by selectively ovipositing eggs in particular site. Before laying the eggs, female may choose the oviposition site by evaluating some features of the food or substrate quality such as plant size, leaf shape, color, odor, taste and presence of other eggs [29].

As well as the importance of host quality before laying eggs, oviposition is also determined by the insect characteristic, such as fecundity. Fecundity is defined as the number of offspring produced by an individual insect that could be determined by the number of eggs produced. Potential fecundity may often be a good indicator of future reproductive output. It was reported that the fecundity of *Anobium punctatum* was affected by the nutritional quality of wood eaten during larval development [30]. Another report also stated that the fecundity of herbivorous insects at both individual and population scale was determined by host plant quality such as carbon, nitrogen, and defensive metabolites [31]. In this chapter, the significance of diet quality other than starch toward oviposition ability and fecundity of the *Lyctus* were discussed.

1.2. Materials and Methods

1.2.1. Insect source

The *Lyctus* used in this study were the adult stage of *L. africanus* from

laboratory colony. The colony was already set upon artificial wood-based diet for about 20 years (T. Yoshimura, personal communication) since the introduction of *L. africanus* in Laboratory of Innovative Humano-habitability, Kyoto University, Japan. The artificial diet used to rear the colony of *L. africanus* is the standard diet for rearing procedure of *L. brunneus* [19]. The colony was maintained in a dark climatic chamber with 26°C and 65% relative humidity (RH). Before conducting the experiment, females and males were segregated based on the morphology of the abdominal terminal segments (see Fig. II.1).

1.2.2. Diet sources

In this experiment, three types of artificial diets were prepared in order to examine an appropriate diet for *L. africanus* beetle. Starch, protein and filler were the main components of the artificial diets. The diets were prepared according to the type of filler used in the diets, as follow: the sapwood portion of *Shorea* sp. in 20–40 mesh size (Diet 1), and two kinds of cellulose materials (cellulose powder [Diet 2] and alpha-cellulose powder [Diet 3]). Both celluloses were obtained from Nacalai Tesque (Kyoto, Japan). For the vital nutrient, soluble starch (Nacalai Tesque) and dried brewer's yeast powder (Asahi Food and Health Care, Tokyo, Japan) were used. The Diet 1 and Diet 2 were the common artificial diets for *L. brunneus* [19], whereas the Diet 3 was an additional diet tested for *L. africanus* in the present study.

1.2.3. Diet preparation

The artificial diets consist of three basic components as described in Table 1.1. The proportion of each component adopted the protocol of *L. brunneus* diet preparation.

Table 1.1. Composition of the three artificial diets for *L. africanus*

Composition	Diet 1 (% w/w)	Diet 2 (% w/w)	Diet 3 (% w/w)
Wood powder	26	–	–
Cellulose powder	–	26	–
Alpha-cellulose	–	–	26
Dry yeast	24	24	24
Starch	50	50	50

Note: w/w is a mass percentage of each diet component in a total mass of diet mixture.

The diets were prepared by mixing the components (Table 1.1) with distilled water, as follow: 70% for wood-based diet (Diet 1) and 90% for each of cellulose-based diet (Diet 2 and Diet 3). Distilled water was useful for preventing cracking or collapse in diet blocks. Each mixed diet was made into dough, and compacted into brick-shaped pellets.

1.2.4. Experiment for diets composition effect on *L. africanus*

The *Lyctus* were raised in three types of diets (Diet 1, Diet 2, and Diet 3). In the experiment, the two blocks of each diet were put into a glass bottle jar (450 ml) with a filter paper Ø 70 mm (Whatman No. 2) at the bottom. Five pairs of newly emerged adults of *L. africanus* were liberated onto the diet and

allowed to oviposit. The jar was lightly capped with a Whatman filter paper No.2 (Ø 70 mm) so as to permit ventilation. It was then stored in a chamber at about 26°C and 65% relative humidity in the dark. Emerging adults from three different diets were used as insect source in the oviposition test.

1.2.5. Preparation of oviposition sites

Quantitative ash less filter papers (Advantec No. 5C, Toyo Roshi Kaisha, Ltd, Japan) were used as oviposition sites. First, the filter papers were figured into square shape with 25 x 75 mm in size, then folded 4 times to form 5 surface layers (Fig.1.1A). For one experimental unit, four slices of folded filter papers were sandwiched with one pair of slide glasses (25 x 75 mm), and then tied up with a paper clip (Fig.1.1B). To attract adult females to lay eggs in the filter paper, a nutritive solution was prepared with 5% (w/v) soluble starch (Nacalai Tesque) and 10% (w/v) sucrose (Nacalai Tesque) in distilled water. The nutritive solution was impregnated into the folded filter paper with an equal weight of the folded filter paper by using a syringe or micro pipette. The folded filter papers treated with distilled water were prepared for control. These experiment units then were dried up at 60°C for 24 hours. After drying up for 24 hours, these experiment units were immediately exposed to *Lyctus* adults fed with three different diets using a no-choice test.

1.2.6. Egg-laying preference / oviposition test

A plastic jar bottomed with an Ø 70 mm circle of filter paper (Whatman No.2) as *Lyctus* footstep was used as a test container. The treated or controlled experiment unit was moved into the plastic jar, and 10 pairs of

adult beetle emerging from different diets were put into the jar (Fig. 1.2A). The plastic jar was covered with gauze to permit ventilation (Fig. 1.2B). All the experiment units were then incubated at 26°C at 65% relative humidity for two weeks.

At the end of incubation time, the number of eggs and survived adults were counted using an objective microscope. Six-replications were employed for each diet.

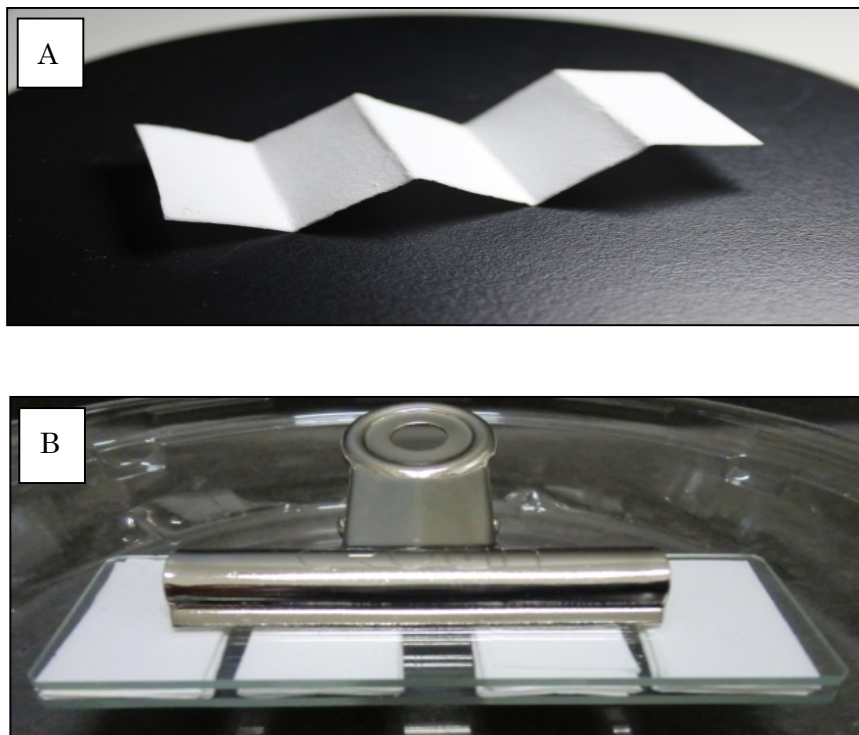


Fig. 1.1. Oviposition sites, folded filter paper (A), ; one experimental unit (B).

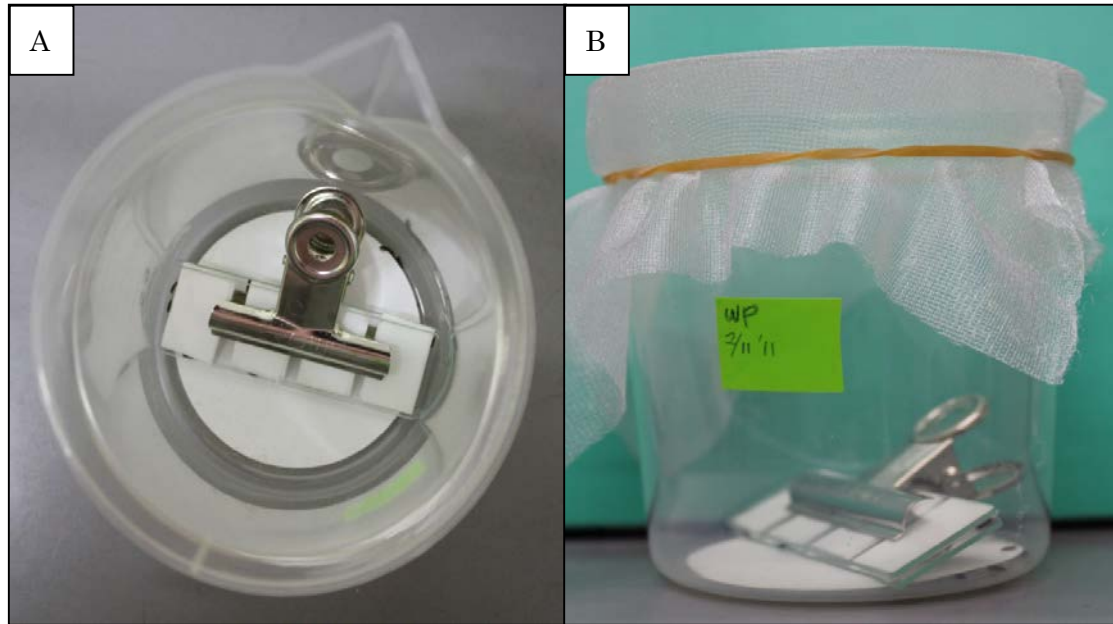


Fig. 1.2. Test method for oviposition experiment, A) One experimental unit inside a plastic jar; B) a plastic jar lid covered with gauze to prevent beetle escape.

1.2.7. Statistical analysis

The data were analyzed by one-way analysis of variance (ANOVA), with the Tukey-Kramer HSD test at 5% critical differences as a post-hoc test.

1.3. Results

Some larvae were eventually hatched from the mature eggs during two weeks. The young and mature eggs were whitish, semi-transparent, cylindrical, and mostly grouped in a cluster (Fig.1.3A, B and C). The eggs were usually oviposited at the edge of filter papers at shallow depth as shown in Fig. 1.3.D.

During two-weeks incubation period, *L. africanus* adults emerged from the cellulose-based diets (Diet 2 and Diet 3) oviposited more eggs significantly ($F = 11.21$; $df = 35$; $p < 0.05$) on nutritive solution- than water-impregnated

filter papers (as shown in Table 2.1), while adults emerged from Diet 1 oviposited fewer eggs than those from both cellulose-based diet. In contrast, all of adults emerged from Diet 1, Diet 2, and Diet 3 laid eggs in lower number on water-impregnated filter papers than those of adults on nutritive solution-impregnated filter papers.

Mostly, the adults emerging from Diet 2 survived significantly in higher number than those of adults from the other diets ($F= 3.94$; $df= 71$; $P < 0.05$). In addition, the number of survived adults for both males and females were not different ($F= 3.941$; $df= 71$; $P < 0.05$). However, males tended to survive in higher number than females.

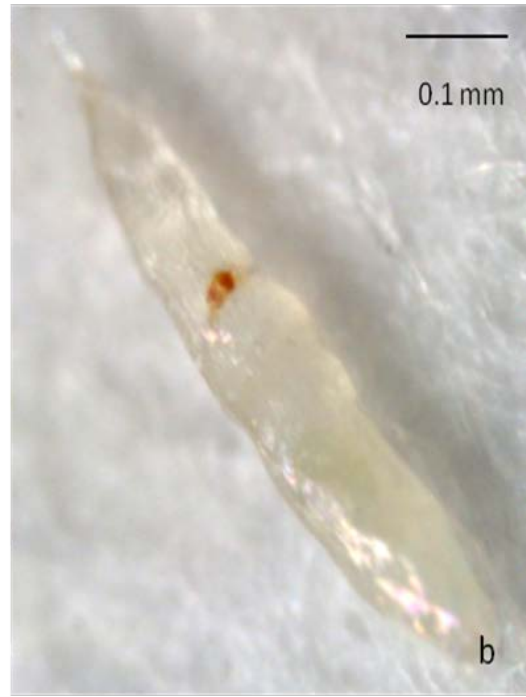


Fig 1.3. Profile of young egg (a), mature egg (b), eggs in cluster (c), and eggs are deposited at the edge of filter paper (d).

Table 1.2. The number of eggs and survived adults of *L. africanus* after oviposition test.

Diets*	Eggs/ 5 females	Survived adults (%)		
		Total adults	Males	Females
WP+	57.50 ^{bc}	45.83 ^{ab}	30.00 ^{abc}	15.83 ^{abc}
WP-	21.00 ^c	35.83 ^{ab}	23.33 ^{abc}	12.50 ^c
CP+	114.33 ^a	59.17 ^{ab}	37.50 ^{ab}	21.67 ^{abc}
CP-	20.83 ^c	65.83 ^a	38.33 ^a	27.50 ^{abc}
AC+	91.67 ^{ab}	34.17 ^{ab}	23.33 ^{abc}	10.83 ^c
AC-	12.83 ^c	27.50 ^b	17.50 ^{abc}	10.00 ^c

Notes: Numbers with the same letter are not significantly different according to Tukey's test ($P < 0.05$).

*: (+) indicates the treatment of filter paper with nutritive solution; while (-) indicates the treatment of filter paper with water.

1.4. Discussion

Generally, copulation occurs shortly (48 hours) after emergence because the new emerged beetles are sexually mature [23,24]. Then, it was followed by an oviposition which occurs during the night after 2 - 4 days of emergence of *L. africanus* [24]. Two-week incubation period was long enough for the egg-laying process of *Lyctus* adults. Hence, some larvae were eventually hatched from the mature eggs during two weeks.

The eggs were usually oviposited at the edge of filter papers at shallow depth. Generally, the eggs are oviposited at 1.0 – 6.5 mm in depth in the wood vessels, preferentially from a transverse surface, but also through radial and tangential faces [32].

In this experiment, combination of starch and sugar in nutritive solution acted as oviposition stimulant for *Lyctus* which attracted all adults emerged from Diet 1, Diet 2, and Diet 3 to lay more eggs. Some studies reported that some chemicals played the role as oviposition stimulant in some insects; for example, leaf epicuticular wax for Far-Eastern knotweed borers (Lepidoptera) [33], aristolochic acids and sequoyitol for butterflies [34], d-catechin for cerambycids [35]. The present result may confirm that starch and sugar are the vital nutrients for *L. africanus* which could attract adult females to lay their eggs. Many studies also reported that *L. brunneus* preferred laying eggs in the starch rich sapwood [25,32,26] after leaving tasting marks on the wood surface to determine the quality of starch. The chemical cues associated with potential host plants guide to selection of suitable site for oviposition [36]. The report also examined that orientation to find a host before ovipositing was mediated by chemical stimuli that might depend on the presence of attractants or the absence of repellents [36].

In this study, combination of starch and sugar took part in attracting for host-finding and oviposition. In addition, the tasting behavior of adult female is sure to support total understanding of the chemical/ecological importance of such nutritive materials. As shown in Table 1.2, the adult insects emerging from cellulose-based diets (Diet 2 and Diet 3) were likely to oviposit more eggs than what Diet 1-emerged adult did. These numbers were lower than that of *L. africanus* [24] and *L. brunneus* [32]. *Lyctus africanus* was reported to oviposit 32 eggs on average in seasoned cuttings of *Poincinia* wood for duration of 1 to 15 days. Though, *L. brunneus* oviposited more than 70 eggs per female over a period of 1-2 weeks. Different host and *Lyctus* species might

have diverse performances in their life trait, including in reproduction. These variances might be affected by host quality influencing survival and reproduction of insect. It has also been reported that host plant quality contributed to different insect performance, including fecundity [37]. Host plant quality described the chemical component of host plant, such as nitrogen, carbon, traced elements, and defensive compounds which could affect the performance of herbivorous insects [31].

One good example of the importance of host plant quality to insect fecundity was the responses of aphids to changes in nitrogen content in Sycamore (*Acer pseudoplanatus*). High content of amino acids in phloem sap had affected the increased responses in fecundity of sycamore aphid [31]. In that report, they also stated that the performance of herbivorous insect was influenced by carbon-based compound as well. The higher the content of carbohydrate compounds, an example of carbon-based compound in an artificial diet, the lower the fecundity in one species of grasshopper (*Melano sanguinipes*). Meanwhile, another species of grasshopper (*Phoetaliotes nebrascensis*) was unaffected [38]. In other words, the carbon-based compound might cause diverse responses even though among closely related species.

In this study, the cellulose-based diets (cellulose is one of abundant carbon-based compounds in nature), both Diet 2 and Diet 3, had a tendency to affect adults stage of *L. africanus* to be more fecund by laying more eggs on filter papers. Basically, all adults emerging from three diets survived in the same values after laying eggs on both oviposition sites. However, adults emerging from Diet 2 survived significantly in higher number than those of

adults from the other diets. Similar findings were also reported on Cerambycidae after evaluating the appropriate diets [39]. The report suggested to utilize the diets combined with cellulose than those with sawdust or phloem-cambium for rearing *Anoplophora glabripennis* since those females of *A. glabripennis* grew faster on that diet—this reason is drawn from the significance of females, in terms of oviposition, to produce offspring as much as possible.

In this study, the diet composition, in the case of cellulose content, was suggested to influence the fecundity of the adult insects of *L. africanus*. It was also reported that nutritional quality of wood influenced the insect fecundity in *Anobium punctatum* [30]. Host quality determined the fecundity of insect and also affected insects reproductive strategies, including egg size and quality, the allocation of resources to eggs and the choice of oviposition sites [31].

In this study, both males and females survived in similar way. However, males tended to survive in higher number than females. It was also reported that the length of survival between males and females of *L. brunneus* was little different [22]. Dissection on the females of *L. brunneus* revealed that the females survived for several days at least after oviposition [22]. It was also found that the female of *L. brunneus* oviposited eggs on the 40th days after the experiment [22]. On the whole, the length of adult's stage of *Lyctus* seems to vary considerably. These diversities might relate to the different strategies to produce vigorous offspring among or even closely related species.

1.5. Conclusions

In this chapter, it was confirmed that starch and sugar are important to attract adult females of *L. africanus* to oviposit the eggs on starchy sites. This study revealed that the female of *L. africanus* emerging from cellulose-based diets was more fecund than those of from wood-based diet which indicated by the number of eggs and survived adults. The cellulose might affect the development of *L. africanus*. However, the significant role of cellulose in *Lyctus* growth need to be confirmed because some previous studies had reported the insignificant role of cellulose in *Lyctus* growth.

1.6. Summary

Oviposition is mostly affected by the quality of food or substrate and also by the fecundity of the insect. In this chapter, the significance of the food or diet quality toward fecundity of the *Lyctus* were discussed.

In experiment, *L. africanus* were raised in three types of diets (wood- [Diet 1], and cellulose- [Diet 2 and Diet 3] based diet). A group of five females and males *L. africanus* were liberated onto the diet and allowed to complete the life cycle. The new generations were harvested and then subjected to oviposition test on a folded filter paper. The number of laid eggs and survived adults after oviposition test were observed.

The results revealed that combination of starch and sugar acted as oviposition stimulant for *Lyctus* adults emerged from Diet 1, Diet 2, and Diet 3. The adults emerging from cellulose-based diets (Diet 2 and Diet 3) were likely to oviposit more eggs than what Diet 1-emerged adult did, which indicated that cellulose might support the oviposition ability of *L. africanus*.

Chapter 2. Rearing and mass culturing of *Lyctus africanus* in laboratory scale

2.1. Introduction

Laboratory studies of *Lyctus* have usually been conducted using an artificial diet instead of wood. *Lyctus* require starch and protein as vital nutrients [25,19]. Therefore, the artificial diets used in *Lyctus* studies have consisted mostly of starch and protein supported with filler or a matrix to enhance the physical properties of the diets. Researchers have developed several breeding methods for *Lyctus* [15,19,20]. Most of the fillers used in the artificial diets have been from the sapwood portion of hardwood, such as *Shorea* sp., in particular combinations with other nutrients to support growth [19,40].

It has been reported that a cellulose-based diet improved the growth of *L. brunneus* [19,20], and that a greater proportion of cellulose in the diet was more suitable for the mass culture of *L. brunneus* than a diet of wood particles (sawdust) in terms of the continuous availability of cellulose as raw material, and the number of emerging adults [20]. It was thought that Lyctinae members are not able to utilize cellulose for their development, thus, cellulose would seem a good choice of filler in an artificial diet of *Lyctus* beetle. In Chapter 1, it was reported females of *L. africanus* emerging from cellulose-based diets tend to be more fecund than those of from wood-powder based diet.

Furthermore, the combination of either *Shorea* sp. sawdust or cellulose powder blended with other nutrients was proposed as an ideal diet for the mass culturing of *L. brunneus* [19]. However, limited results on mass

culturing of *L. africanus* with artificial diets have been reported. In 1962, a study on *L. africanus* reported that this beetle was successfully bred on an artificial diet, a mixture containing 90 parts wheat flour and 10 parts yeast [15]. Unfortunately, there was no information on the colony size of the beetle with this artificial diet.

In this chapter, the usefulness of several fillers proposed by Iwata and Nishimoto (1983) in artificial diets for *L. brunneus* to improve the growth of *L. africanus* was evaluated. The use of wood as a filler may be problematic for the mass culturing of *Lyctus* beetles in light of its availability, time-consuming preparation, and the varying distribution of nutrient substances in different pieces and parts of wood, even within the same log. In contrast, cellulosic materials are commercially available. Here, the utility of wood- and cellulose-based diets for *L. africanus* by examining some parameters in insect fecundity was discussed. In addition, by clarifying the favorable diet conditions for *L. africanus*, we discuss the possibility of obtaining greater numbers of *Lyctus* adults in laboratory settings for further study.

2.2. Materials and Methods

2.2.1. Insect sources

Adult-stage *L. africanus* bred in a laboratory colony were used for this study. The colony was already set up on artificial wood-based artificial diet. The artificial diet used to rear the colony of *L. africanus* is the standard diet for rearing procedure of *L. brunneus* [19]. The colony was maintained in a dark climatic chamber with 26°C and 65% relative humidity (RH).

2.2.2. Diet sources

In this experiment, three types of artificial diets were prepared in order to examine an appropriate diet for *L. africanus* beetle. Starch, protein and filler were the main components of the artificial diets. The diets were prepared according to the type of filler used in the diets (see Chapter 1).

2.2.3. Diet preparation

The artificial diets consist of three basic components as described in Chapter 1. The proportion of each component adopted the protocol of *L. brunneus* diet preparation.

In this study, two different sizes were used for the diets; 1) for the larval stage observation (larval number), 12 g of a mixed diet was prepared into bricks 2 x 2 x 2 cm in size; 2) for the adult stage observation (total population, sex ratio, and body weight), 100 g of a mixed diet was pressed into bricks 8 x 4 x 2 cm in size. The pressed diet was oven-dried at 60°C for 3 days to prevent attack by fungi, then stored in a plastic box at 26°C and 65% relative humidity in the dark.

2.2.4. Total larvae (immature-stage)

Two servings of the diet (2 x 2 x 2 cm) were placed in a closed Petri dish (125-mm dia., 30 mm height) covered with filter paper (125-mm dia., Whatman No. 2, GE Healthcare, UK) and were maintained in dark climatic chamber at 26°C and 65% relative humidity (RH). Five pairs of newly emerged males and females *L. africanus* were introduced into the dish. The number of larvae in the dish was observed after 45-50 days, before new adults

emerged. The larvae were collected after the artificial diet was immersed in water. The floating larvae were harvested and counted to estimate the oviposition potential of the adult *L. africanus* beetles. This experiment was replicated six times.

2.2.5. Population, sex ratio and body weight of newly emerged adults

Two servings of the diet (8 x 4 x 2 cm) were put in a glass jar (450 mL with 65 mm., 125 mm height) with a filter paper (70-mm dia., Whatman No. 2, GE Healthcare, UK) on the bottom. Five pairs of newly emerged males and females *L. africanus* were introduced into each jar and allowed to oviposit. The jar was lightly capped with a filter paper (70-mm dia., Whatman No. 2, GE Healthcare) to permit ventilation. The jar was then stored in a dark climatic chamber (26°C and 65% RH) until new progeny emerged. Eight replications of each experimental unit were completed.

The emerged adults were harvested and then observed the total population, sex ratio, and body weight. The observations were conducted at two-day intervals throughout the emergence period. Total population was obtained by summing up all of newly emerged adults during the emergence period. The sexes of each beetle were determined by the presence or absence of a heavy fringe of hair along the hind margin of the abdominal sternite: adult females have this fringe (see Fig. II.1.). The sex ratio was defined as the ratio of the number of males to females. Body weight was measured by weighing all of the harvested adults. The body weight of a single adult was estimated by calculating the average body weight considering the number of harvested adults.

2.2.6. Statistical analysis

The obtained data were analyzed by one-way analysis of variance (ANOVA), with the Tukey-Kramer HSD test at 5% critical difference as a post-hoc test.

2.3. Results

2.3.1. Larval number

The average number of harvested larvae was not significantly different among the diets ($F = 0.45$, $df = 2$, $P = 0.65$) which mean the adult fecundity did not differ among the diets, as follow: 141.0 for the Diet 1, 134.2 for the Diet 2, and 113,8 for the Diet 3 (as shown in Table 2.1).

Table 2.1. Average number of larvae *L. africanus* in three artificial diets

Artificial diet	Total larvae/5 female (mean \pm S.E.)
Diet 1	141.0 \pm 10.41 a*
Diet 2	134.2 \pm 24.48 a
Diet 3	113.8 \pm 25.00 a

Note: *Same letter is not significantly different (Tukey-Kramer HSD test; $P < 0.05$) following one-way ANOVA.

2.3.2. Total adults

The growth of *L. africanus* in the three diets showed a similar pattern. The first emergence of a new generation was occurred after 10 weeks of incubation period (Fig. 2.1A). The emergence phases lasted about 4 – 5 weeks (Fig. 2.1B) in all three diets, followed by a dormant phase of about 4 weeks

before the next cycle of the second generation began. Thus, the observation time for the first generation took 15 weeks or 3.5 months. The number of emerging beetles increased in the first two weeks, and then slowly decreased to the dormant phase, which began at the sixth week, as shown in Fig.2.1. New adult *L. africanus* beetles on the Diet 1 and Diet 2 emerged five days earlier than that on the Diet 3 (Fig.2.1B). The number of emerging adults increased in the first two weeks, and then slowly decreased to the dormant phase, which began at the sixth week.

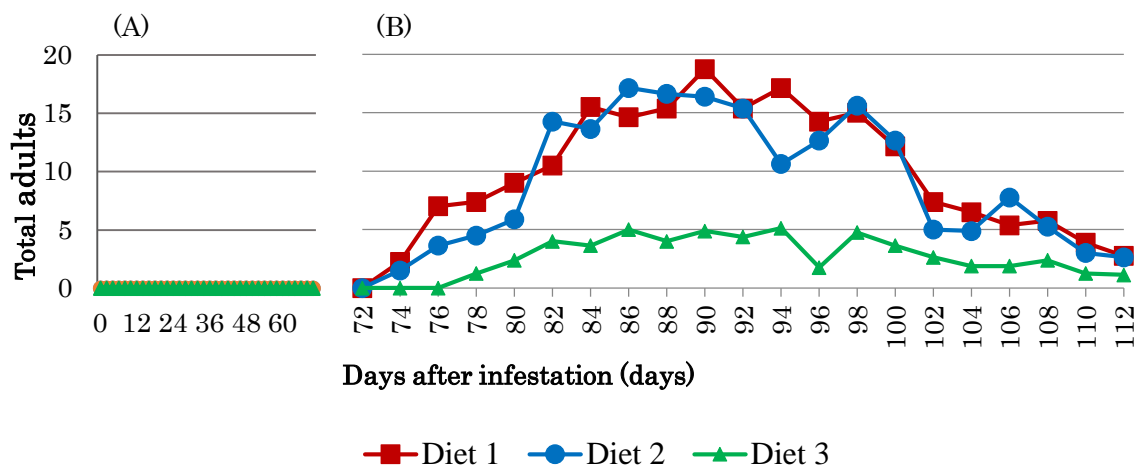


Fig. 2.1. Growth pattern of *L. africanus* on different diets (Diet 1: wood particle-based diet; Diet 2: cellulose-powder based diet; Diet 3: alpha-cellulose-based diet) which shows the incubation (A) and emerging (B) period.

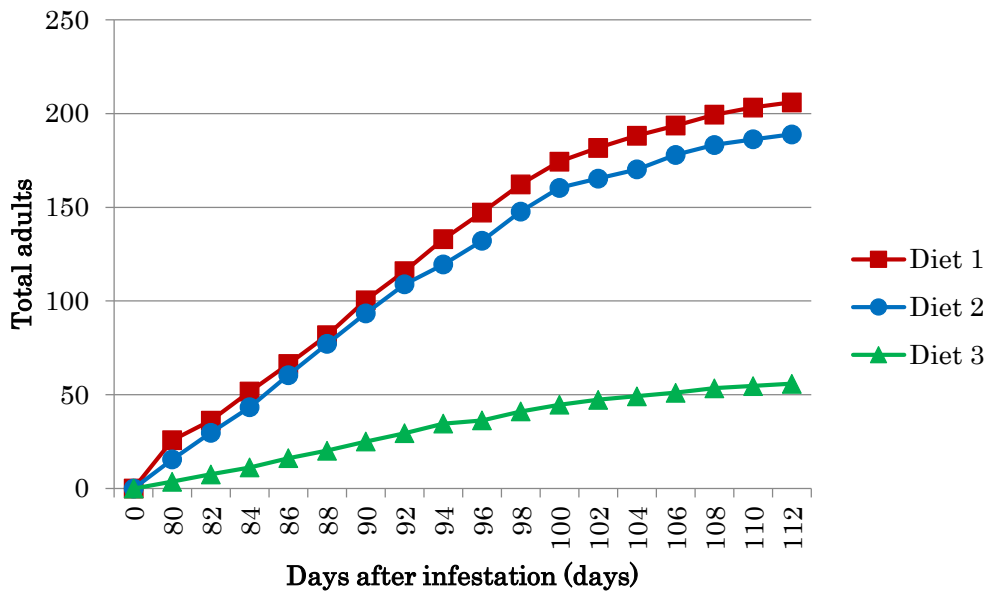


Fig. 2.2. Average cumulative number of emerged adults of *L. africanus* fed with different diets.

To evaluate the ability of adult *L. africanus* beetles to develop with the diets, total population was calculated by summing-up all the adults harvested from each jar at the end of observation (Fig.2.2).

The total populations from Diet 1 and Diet 2 were significantly bigger than those on the Diet 3 ($F = 5.57$, $df = 2$, $p = 0.011$). The Diet 1 and Diet 2 generated 206 and 188.9 total adults, on average, respectively, while the Diet 3 produced only 55.9 on average.

2.3.3. Sex ratio

The sex ratios of the emerged adults from the Diet 1, Diet 2 and Diet 3 are shown in Table 2.2. The adult females tended to be produced in higher numbers than adult males in every diets. The sex ratios were 0.94 ± 0.06 for Diet 1, 0.80 ± 0.05 for Diet 2 and 0.76 ± 0.08 for Diet 3, meaning that the adult

females were produced in higher numbers than adult males. The data analysis of the sex ratios did not show any significant difference among the three diets ($F = 2.27$, $df = 2$, $p = 0.128$).

In wood-based diet (Diet 1), males outnumbered the females at the first emerging time (0-6 days on average), and then replaced by females outnumber the males until the end of observation (Fig. 2.3A.). A different trend occurred in cellulosed-based diets (Diet 2 and Diet 3). In these diets, females predominantly outnumbered the males throughout the emergence period (Fig.2.3B,C).

2.3.4. Body weight

The three diets did not affect the body weight of newly emerged adults of *L. africanus* ($F = 0.32$, $df = 2$, $p = 0.731$). The average body weights of *L. africanus* fed on the Diet 1, Diet 2 and Diet 3 were 1.98 ± 0.04 mg, 1.87 ± 0.06 mg, and 2.01 ± 0.21 mg, respectively; there was no significant differences between them (Table 2.2).

Table 2.2. Development of *L. africanus* in three artificial diets

Artificial diet	Total adults	Sex ratio (M/F)	Body weight (mg)
Diet 1	206.0 ± 49.37^a	0.94 ± 0.06^a	1.98 ± 0.04^a
Diet 2	188.9 ± 30.60^a	0.80 ± 0.05^a	1.87 ± 0.06^a
Diet 3	55.9 ± 16.18^b	0.76 ± 0.08^a	2.01 ± 0.21^a

Note: a.b significant differences in the same column by Tukey-Kramer HSD test ($P < 0.05$).

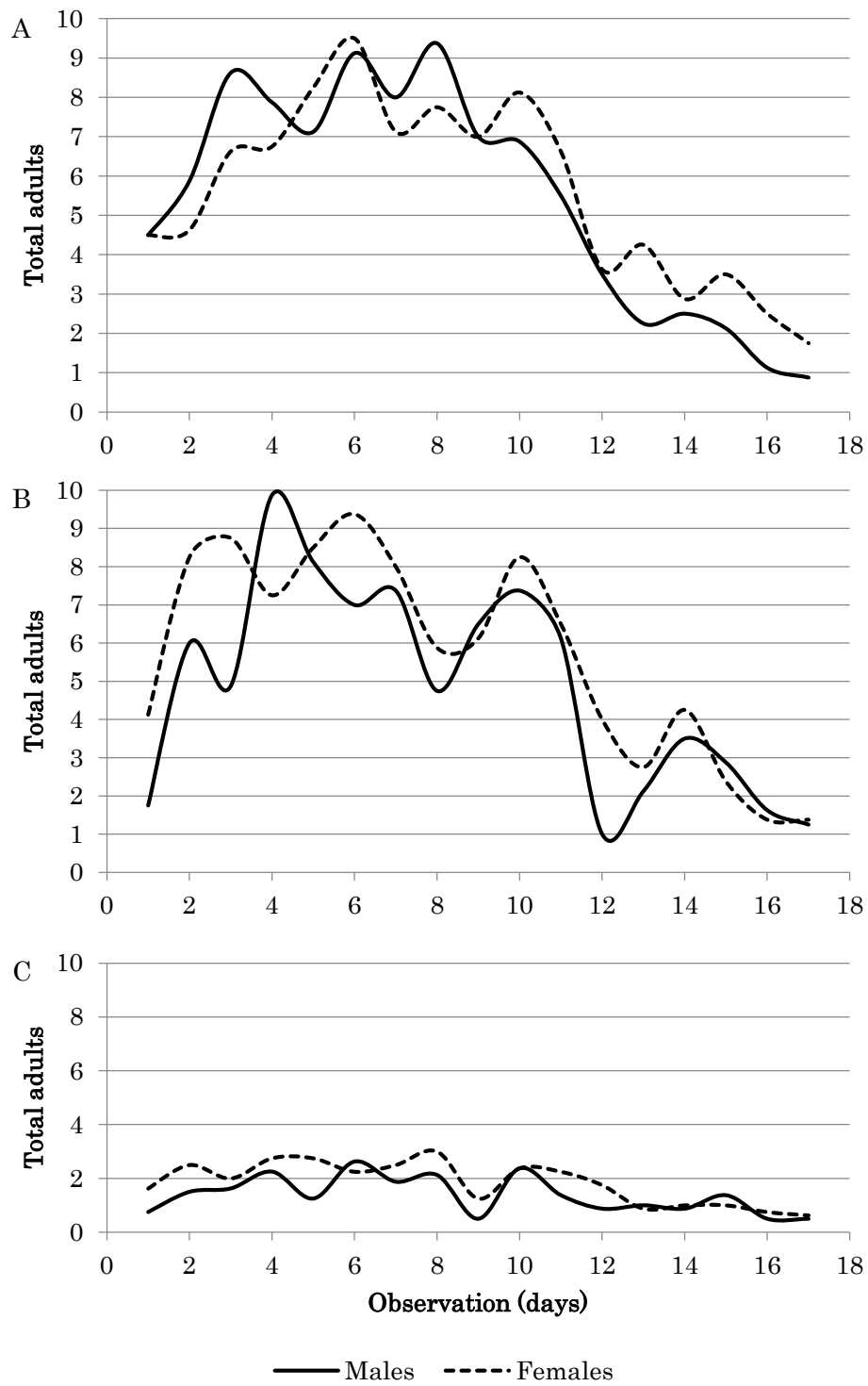


Fig. 2.3. Number of males and females of *L. africanus* fed with A) Diet 1, B) Diet 2, and C) Diet 3.

2.4. Discussion

Rearing Bostrichidae on an artificial diet may shorten the developmental growth and enlarge the number of progeny of these beetles. A previous study of *L. africanus* also reported shorter periods of incubation with an artificial diet rather than wood veneers [15]. Cakes of whole wheat flour were described as a possible substitute for rubber wood in the culture medium of *Heterobostrychus aequalis* (Bostrichidae) [41]. In that study, the diet with whole-wheat cakes produced five times more progeny than the diet based on rubber wood blocks. A study of the biology of *L. brunneus* reared on *Alstonia scholaris* sapwood at various temperatures found that an increase of the incubation room temperature reduced the length of the developmental period by about four months, with optimization at 26°C [32]. In artificial diets, the growth period of *L. brunneus* was also about four months [20]. Another study on *L. africanus* mentioned that the optimum temperature for rearing *L. africanus* beetles on wood pieces was 30°C at 55-65% relative humidity, resulting in a shorter generation period of 2 – 3 months [42]. In the present study, the developmental period of *L. africanus* with the artificial diets at 26°C took about 3.5 months for the first-generation period.

In this study, insect fecundity was used as a consideration for selecting the most suitable diet in order to obtain sound mass culture of *L. africanus* in the laboratory. Awmack and Leather (2002) reported that the nutritional component of diet absolutely affected insect performance and fecundity [31]. Here, fecundity was defined as the actual number of offspring produced by the insect, thus number of total larvae, total adults, sex ratio, and body weight were measured.

The results showed that the number of larvae hatched with each diet was similar, suggesting that the adult *L. africanus* can oviposit without preference on Diet 1, Diet 2 and Diet 3. However, the number of hatched larvae might not reflect the number of adults exactly, because the adults or progeny produced were affected by host quality during both the larval and adult stages [31]. Regarding of total adults data, all three diets allowed a complete life cycle of *L. africanus*. The periods from oviposition to adult emergence in this study were similar to previous study [15]. Nevertheless, significantly higher population of adults were obtained on Diet 1 and Diet 2 than was in Diet 3 though the fecundity did not differ among the diets. It has been reported that wood-based (Diet 1) and cellulose-based diets (Diet 2) improved the growth of *L. brunneus* [19,20]. In terms of the continuous availability of cellulose as raw material, the greater proportion of cellulose in the diet was more suitable for the mass culture of *L. brunneus* and *L. africanus* than a diet of wood particles (sawdust) [20] because of its easy handling and time-saving preparation. The chemical grade cellulose powder also contain a little amount of hemicellulose and other substances. The digestive fluid of *L. brunneus* might partially hydrolyzed the polysaccharides which are intermediate in composition between starch and the hemicelluloses [43]. Contrary, the Diet 3 did not improve the growth of this *Lyctus*. The physical and/or chemical properties of alpha-cellulose fiber in the Diet 3 might impede the development of the larvae on this diet due to the attribute of alpha-cellulose fiber. Alpha-cellulose is considered a strong or vigorous fiber, because its anhydroglucose chains have not been substantially degraded [44]. Furthermore, alpha-cellulose used in this study contained mannose (3.4%) and rhamnose (1.3%), as well as glucose

(78.9%) and xylose (13.2%), indicating an origin of hardwood and softwood mixture. Meanwhile, the cellulose powder only consisted of glucose (76.2%) and xylose (10.7%) which was originated from only hardwood (T. Takano, unpublished data). The *L. africanus* might not utilize the mannose and rhamnose which are known as a constituent of hemicellulose in softwood.

The sex ratio results revealed that the ratios of adults in all three diet groups were lower than 1.0, indicating that adult females were produced in slightly higher numbers than adult males. Most lyctine studies have reported sex ratios approximately equal to 1, and females slightly outnumbered males in sapwood pieces [32,26,23]. With artificial diets, the sex ratio of *L. africanus* and *L. brunneus* were also equal to 1 [19,45]. According to those data, the female survival rate from larva to adult might higher than the male on the diets in this study. Altson reported that the number of female *L. brunneus* was higher than males: thus, one male fertilized several females [23]. Sex ratio might be a fundamental issue for maintaining the mass culture of insects. With regard to the importance of females and males in a population, it might be good to have more females than males to produce as many offspring as possible. In addition, a higher number of males than females in a population could reduce the oviposition potential in females because of male competition [39]. To the best of our knowledge, the importance of sex ratio in lyctinae has not yet been reported in detail.

In addition, the males harvested from the wood-based diet were observed outnumbering the females at the first emerging time, and then the females outnumbered the males until the end of the observation period. In the present study, the number of females on diets containing cellulose

materials (Diet 2 and Diet 3) outnumbered males considerably at the first emerging time, and this ratio relatively continued until the last emerged adult. The sex ratios of emerged adults on both cellulose-based diets at the end of observation were quite low (0.83 and 0.75, respectively). It was also occurred on *L. brunneus* reared in Diet 2 (0.89) [8]. A study reported that the female Asian long horned beetles (*Anoplophora glabripennis*, Coleoptera: Cerambycidae) grew faster on a cellulose-containing diet compared to females fed a poplar tree sawdust-containing diet [20]. In that study, the males fed the cellulose-containing diet grew more slowly than the males fed the diet containing sawdust [20]. We suspect that in our present study, the cellulose might have slightly influenced the females' fitness. One report had been mentioned the presence of cellulase in *Lyctus* spp. [46]. However, the significant function of cellulose in *L. africanus* life span is still uncertain.

The body weight of *L. africanus* emerged from the three different diets were similar. When rearing *L. brunneus* on sawdust- and cellulose-based diets (equivalent to Diet 1 and Diet 2 in this study), Iwata and Nishimoto [19] used body length as one of the parameters for body weight, and reported that the beetles reared on sawdust- and cellulose-based diets produced similar, well-developed (large) individuals. They also mentioned that there were no significant differences in body length among individuals reared on each diet. The present results suggest that the Diet 1, Diet 2 and Diet 3 did not affect the food consumption of larval-stage *L. africanus*. In the case of other insects fed on low-quality foods, insects compensated for inadequate nutrient uptake by eating more food, which enabled them to attain their proper body weight [37,47].

As mentioned above, larvae number, sex ratio, and body weight of *L. africanus* were similar among the three diets. However, the total number of new adults was significantly lower in the Diet 3. Based on our results, we concluded that the filler used in artificial diets for *L. africanus* seems to affect diet performance, which influenced the beetles' growth. The amount of vital nutrient (starch) in the diet is not the only important factor to be considered when selecting a suitable diet for *L. africanus*. The filler should also enhance oviposition potential and larval development [48]. In addition, the density of the artificial diet is an important factor influencing lyctines' growth [20,49]. Utilization of alpha-cellulose as a filler of artificial diet for rearing *Lyctus* is not proposed due to its fiber characteristics.

For rearing *L. brunneus*, Diet 1 and Diet 2 could be used alternately [19]. However, it was reported that malformations were found occasionally in the abdominal segmentation of other *Lyctus*, *L. brunneus*, beetles that emerged from diets without sawdust [19]. These malformations were likely generated by the absence of some necessary compounds in cellulose-based diets [19], such as linoleic acid and sterols [50], steroid and minerals that are present in wood [51]. However, the malformation effect on *Lyctus* development is unclear. As a result, utilization of Diet 2 (as a non-sawdust diet) in mass culturing of *L. africanus* is possible when sawdust or wood particles are not available.

In the next chapter, aggregation behavior of *L. africanus* was studied on a newly emerged adults of *L. africanus* from Diet 1.

2.5. Conclusions

Rearing Bostrichidae on an artificial diet may shorten the developmental growth and enlarge the number of progeny of these beetles. The life cycle of *L. africanus* was shortened in wood- and cellulose-based artificial diets. All three diets allowed a complete life cycle of *L. africanus*. However, the alpha-cellulose based diet could not support the growth of *L. africanus*.

For rearing the *L. africanus*, wood-based (Diet 1) and cellulose-based diets could be used alternately. However, the utilization of cellulose-based diet (Diet 2) should be utilized for mass culturing of *L. africanus* when sawdust or wood particles are not available.

2.6. Summary

The usefulness of several fillers used in artificial diets of *L. brunneus* were evaluated to improve the growth of *L. africanus*. The use of wood as a filler may be problematic for the mass culturing of lyctine beetles in light of its availability. In contrast, cellulosic materials are commercially available.

In this study, three types of artificial diets (Diet 1, 2 and 3) were prepared to rear *L. africanus*. The newly emerged beetles were harvested and observed the number of larvae, adults, sex ratio, and body weight.

The results indicated that total larvae, sex ratio, and body weight of *L. africanus* were similar among the three diets. However, the total adults was significantly lower in the Diet 3. It was suggested that the amount of vital nutrient (starch) in the diet is not the only important factor to be considered when selecting a diet for *L. africanus*. The filler should also enhance oviposition potential and larval development. Hence, Diet 1 and Diet 2 could

be used alternately for rearing *L. africanus*. However, utilization of Diet 2 for mass culturing of *L. africanus* was suggested when sawdust or wood particles are not available.

V. Part II. Aggregation pheromones of *Lyctus africanus* Lesne
and its application in semi-field

Chapter 3. Screening of the potent chemical compounds in aggregation behavior of *Lyctus africanus*

3.1. Introduction

Lyctus damage is inconspicuous in the field, thus, infestations are usually discovered belatedly due to the difficulty of locating and monitoring them. Once the infestations occur, it will develop creating the obscured galleries inside the infested materials. In early stage of infestation, visual signs of the infestation are invisible. Consequently, the degree of *Lyctus* infestations are unknown, hence their population is unpredicted.

To estimate the *Lyctus* population in its natural habitat, it is necessary to examine the degree of infestation. Some monitoring techniques have been developed to regularly inspect insect populations. The use of insect traps for monitoring has been widely used in pest management [16]. Traps must enable insects to be detected at very early stage of infestation that minimizes and improves the prevention of damage. In addition, insect monitoring involves detecting insect numbers over time. Generally, insect traps are combinations of attractants and killing systems. Some attractants are widely used, including food baits, pheromones, light and different colored panels and sections on exterior and interior surfaces [17]. Unfortunately, there is still no device for detecting and monitoring *Lyctus* beetles in the field due to the lack of sufficient data on their ecological features. Thus, strategies for monitoring and controlling this beetle by examining the *Lyctus* ecology are urgently needed.

Limited studies have been carried on *Lyctus* ecology although one

preliminary study on *L. brunneus* reported that males and females were intensively attracted by females` odor [52]. Unfortunately, there was no further information to reconfirm whether the attraction activity is due to such a pheromone. Based on the results, the *Lyctus* was suggested to possess an aggregation behavior elicited by chemical signals. In this chapter, chapter 3, we evaluated the behavior of *L. africanus* in the laboratory by screening the potent chemical compound for aggregation responses in *L. africanus*.

Studying the aggregation behavior of *Lyctus* beetles is crucial to understanding its ecological aspects. The behavior was investigated using a chemical approach through comprehensive screening of the potential compound produced by *L. africanus*. This study will contribute significantly to our understanding of the *Lyctus* ecology for the purpose of developing monitoring techniques for *Lyctus* beetles.

3.2. Materials and Methods

3.2.1. Insect colony

For behavioural and chemical analytical investigations conducted in this study, only the adult *L. africanus* were used. The beetles were obtained from laboratory sources maintained at the Research Institute for Sustainable Humanosphere, Kyoto, Gokasho, Uji, Japan. The larval beetles were reared on wood-based artificial diet (the compositions are described in Chapter 1). The wood-based artificial diet was used to conduct further study on aggregation behaviour of *L. africanus* due to its similar characteristics with sapwood of hardwood species as natural diet for *Lyctus*.

The *L. africanus* cultures were maintained in glass jars (450 mL) at a sealed chamber at constant temperature (26 ± 2 °C) and relative humidity ($65 \pm 10\%$). The new emerged adult female and male beetles were sexed and separated under a dissection microscope (see Fig. II.1).

3.2.2. Collection of chemical compound

To collect the chemical compound, whole body extractions using hexane solvent on newly emerged beetles were performed similar methods previously reported for other beetle and millipede groups [53-55]. Newly emerged adults beetles were defined as 1-7 days old. The newly emerged *Lyctus* are sexually mature upon emergence [23,7]. Fifty adults for each sex were later inserted in individual polyethylene test tubes (2-mL self-lock Eppendorf tube, Germany) and 1000 μ L of hexane was added and left for 24 hours in a constant environmental chamber and transferred obtained extract into a new empty polyethylene tube before further processing.

3.2.3. Behavioral activity

We conducted bioassays to measure responses of *L. africanus* to crude extracts from exterior chemical washes of adult beetles. The behavioral experimental used were an adaptation of the dual-choice bioassays [53,56]. Bioassays were conducted in a closed Petri dish (\varnothing 90 mm) with filter paper (\varnothing 90 mm, Whatman No. 1, GE Healthcare, UK) at the bottom. Two small paper discs (\varnothing 10 mm, 60 mg, Advantec type 27, Toyo Roshi Kaisha, Japan) were used to deposit liquid aliquot solutions containing either the treatment substance or the control. Each small paper discs within Petri dishes were

saturated with 50 μL , the equivalent 2.5 beetles, of either male or female crude extract. The same volume of hexane was applied to the control paper disc, and the discs were then air-dried for approximately 1 min before the testing. Each pair of paper discs was placed in a Petri dish, with the discs placed opposite one another (60 mm in distance). Prior to observations, the positions of paper discs within dishes were randomized. A group of twenty *L. africanus* beetles, both females and males, were placed in the middle of the dish at the far edge where vertical and horizontal surfaces meet (Fig. 3.1.).

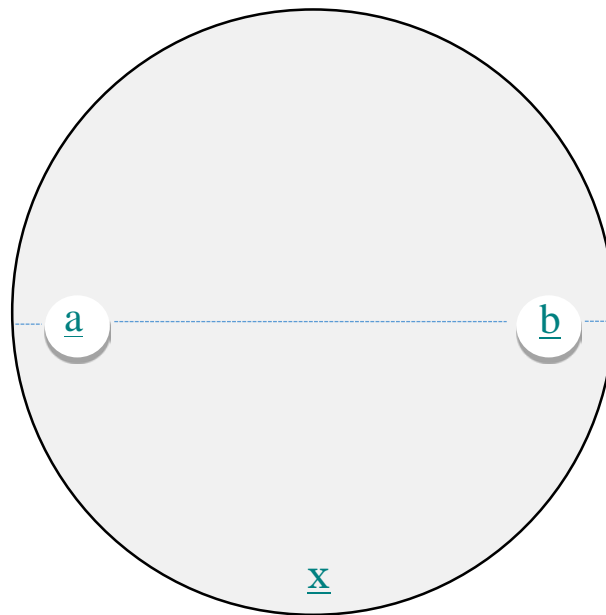


Fig. 3.1. The experimental design of dual-choice bioassays in a 9 cm Petri dish; (a) and/or (b) two small paper discs for control and treated-one, (x) starting/releasing point of beetles.

This arrangement in the dishes allowed all beetles to have the same starting point; thus the distance from the starting point to the small discs was the same. The number of beetles that aggregated on either of the paper discs was recorded using a video recorder (Sony, model. HDR-XR 500 V, Japan),

which observed the dish and contents for a period of 5 min. Beetles settling apart of the control and test discs were scored as nonresponders. All observations of beetles were conducted in a dark chamber under a red light illumination with constant temperature and humidity.

3.2.4. Chemical analysis

GC–MS was performed to identify specific chemical compounds in the crude extracts that induced the aggregation of beetles on paper disks. Extracts were prepared by the immersion of a single adult beetle in hexane (10 μ L) for 5 min and injected into a GC–MS instrument. The instrument used was a Network GC System (6890N; Agilent Technologies, USA) coupled with a mass selective detector (5975 Inert XL; Agilent Technologies) operated at 70 eV. The column used was an HP-5ms capillary column (Agilent Technologies, 0.25-mm I.D. \times 30 m, 0.25- μ m film thickness). The carrier gas was helium, with a constant flow rate of 1 ml/min. Samples were analyzed in the splitless mode with the temperature programmed to change from 60 $^{\circ}$ C (initially for 2 min) to 290 $^{\circ}$ C at a rate of 10 $^{\circ}$ C/min. The final temperature (290 $^{\circ}$ C) was then maintained for 5 min. The GC–MS data were recorded using Chemstation (Agilent Technologies) with reference to an MS data base (Agilent NIST05 mass spectral library, Agilent Technologies). Column chromatography was performed on a Wakosil silica gel C-200 column with the specified solvents. 1 H- and 13 C-NMR spectra were recorded on a Bruker Biospin AC400M spectrometer (400 MHz for 1 H and 100 MHz for 13 C), using tetramethylsilane as the internal standard.

3.2.5. Purification and isolation of chemical compounds

Purification of chemical compounds was performed by serial extraction. First, the male crude extract was prepared in the hexane solvent. One hundred male beetles were transferred into a clear polyethylene tube (a 2 mL self-locking Eppendorf tube), and immersed in 2 ml hexane for 24 h. The resulting extracts were filtered through Whatman No.1 paper, concentrated in vacuo, and applied to an SiO₂ column (0.5 g, Wako-gel C-200), shown in . The column was successively eluted with hexane, three mixtures of ethyl acetate (EtOAc) in hexane (10, 20, and 50%), and EtOAc (5 mL each). All fractions, then known as Fr.1, Fr.2, Fr.3, Fr.4 and Fr.5 for each fraction of hexane, three mixtures of ethyl acetate (EtOAc) in hexane (10, 20, and 50%), and EtOAc, were subjected to GC–MS analyses for identification and were concentrated to 2-ml aliquots for bioassay to confirm the activity of each fraction.

3.2.6. Identification and syntheses of the chemical compounds

Identification of chemical compounds were conducted by GC–MS instrument. The GC–MS data were recorded using Chemstation (Agilent Technologies) with reference to an MS data base (Agilent NIST05 mass spectral library, Agilent Technologies). Structural determinations of the compounds were conducted by the comparison of GC retention times of the candidate structures with natural compound. On the GC–MS analysis, synthesizing the synthetic compounds are required to analyse the complex chromatographic data of the natural and synthesized compounds. The synthesized compounds structurally similar in terms of chromatographic

retention time and mass spectral appearance. As well as, co-chromatography of the synthetic compound with natural compound was also performed to identify the chemical compounds. The synthetic chemical compounds were synthesized in Faculty of Bioenvironmental Science, Kyoto Gakuen University, Japan.

3.2.7. Quantitative determination of three esters

To determine the quantity of each ester in a male *Lyctus*, a calibration curve was constructed for each compound. The curve was obtained by correlating GC–MS response data of male crude extract with each concentration of three standard solutions. The male crude extract was prepared by dipping one male *L. africanus* into hexane (10 μ L) and was then subjected to extraction for 5 min. The extract was collected with a 10- μ L microsyringe and subjected to GC–MS analysis ($n = 9$ trials). A synthetic compound of each ester (2-propyl dodecanoate **1**, 3-pentyl dodecanoate **2**, and 3-pentyl tetradecanoate **3**) was diluted with hexane. The following concentrations were prepared: 5, 10, and 25 ng/ μ L; and a 200 ng/ μ L solution was also prepared for the major compound (**2**). A calibration curve was then constructed.

3.2.8. Statistical analysis

Ten replicates were made for each treatment combination of either crude extract or fractions. The counts of beetles preferring either treated or untreated (control) paper discs were scored after 5 min. of observation and analyzed for significant differences. Wilcoxon matches pairs test was

conducted to compare the total number of *L. africanus* beetles on either treated or control disk. To determine the responses of *L. africanus* beetles with regard to each crude extract, an aggregation index (AI) value was calculated as [57-60]:

$$\text{Aggregation Index (AI)} = \frac{(T-C)}{N} ,$$

where *T* is the number of beetles located on the treated paper disk.

C is the number of beetles located on the control disc.

N is the total number of beetles used in the bioassay.

The index value ranged from +1 to -1. Only the positive value of AI corresponds to an aggregation response. Means of AI values of each crude extract over 5 min. observations were transformed into log transformation and submitted to one-way analysis of variance, followed by Tukey's Honestly Significant Differences Test (HSD) test ($\alpha = 0.01$). Student *t*-test ($P < 0.05$) was performed to compare the responses of female and male beetles exposed to each crude extract. All of data analysis were performed using JMP®, version 9; SAS Ins.

3.3. Results

3.3.1. Behavioral activity

Based on laboratory testing and recorded visual observations, *L. africanus* showed an aggregative behavior. Within minutes of being placed and released in Petri dishes, *L. africanus* adults exhibited searching behavior, moving around the dish, and finally formed a group or cluster. Treatment replications for adult, included crude extracts for both males and females in three combinations; male crude extract (ME) vs. control, female crude extract (FE) vs. control, and ME vs. FE. The beetles also aggregated immediately on the preferred disc. There was a significant preference of both female and male beetles for settling on the ME-treated paper discs rather than control paper discs (Table 3.1). Furthermore, the responses of the *L. africanus* beetles against each crude extract were illustrated as an aggregation index (AI) shown in Table 3.2. The AI indicated that the both female and male beetles preferred the ME-treated paper discs to the FE-treated discs (female: $F = 50.72$, $df = 29$, $P < 0.0001$; male: $F = 7.50$, $df = 29$, $P < 0.0001$). However, female beetles showed a higher preference than the males for the ME-treated paper discs (Student t -test, $P < 0.05$). On the other hand, FE-treated paper discs showed insignificant responses for both male and female beetles. This result suggest that the ME contain particular compounds triggering aggregation behavior in *L. africanus*.

Table 3.1. Responses of adult *L. africanus* to discs treated with male (ME), female (FE), and control, indicated by total number of beetles in percentage ($N = 20$; $n = 10$)

Treatment	Tested beetles	% responder beetles		<i>P</i> value
		Treated	Control	
ME vs. Control	♀	64.00 ± 3.63	1.95 ± 0.57	0.002*
	♂	42.30 ± 3.40	6.40 ± 1.09	0.002*
FE vs. Control	♀	22.80 ± 4.51	16.00 ± 3.08	0.432
	♂	15.05 ± 2.98	11.10 ± 1.81	0.223
		ME	FE	
ME vs. FE	♀	53.10 ± 3.42	2.75 ± 0.72	0.002*
	♂	32.70 ± 5.11	4.35 ± 1.45	0.002*

Notes: Level of significance difference for number of beetles on disc, shown by asterisk symbols (Matched pairs test).

Table 3.2. Aggregation index of adult *L. africanus* responses to treated discs with male (ME), female (FE) crude extract and control (50 μ L=2.5 beetles equivalent) ($N=20$; $n=10$)

Tested beetles	Treatment		
	ME vs. Control	FE vs. Control	ME vs. FE
♀	0.62 \pm 0.04 ^{a(*)}	0.07 \pm 0.06 ^{b(ns)}	0.50 \pm 0.03 ^{a(*)}
♂	0.36 \pm 0.04 ^{a(*)}	0.04 \pm 0.02 ^{b(ns)}	0.28 \pm 0.06 ^{ab(*)}

Notes: Aggregation index value (mean \pm SEM), which indicates the significance difference ($p < 0.01$) by different letter within treatments (for each female and male) according to Tukey's HSD test. The letter in parentheses refers to the comparison between female and male beetles in the same treatment (Student' *t*-test). *: Significant; ns, not significant.

3.3.2. Chemical analysis

Crude extracts from males and females were compared using GC-MS analysis (Fig. 3.2). Three peaks (**1**, t_R 14.92 min; **2**, t_R 16.95 min; and **3**, t_R 18.95 min) were detected as male-specific components. In contrast, no trace of the corresponding peak was found in the extracts from the females. Other compounds (pentacosane, C_{25} ; heptacosane, C_{27} ; nonacosane, C_{29} , and hentriacontane, C_{31}) were distributed in both males (in small amounts) and females.

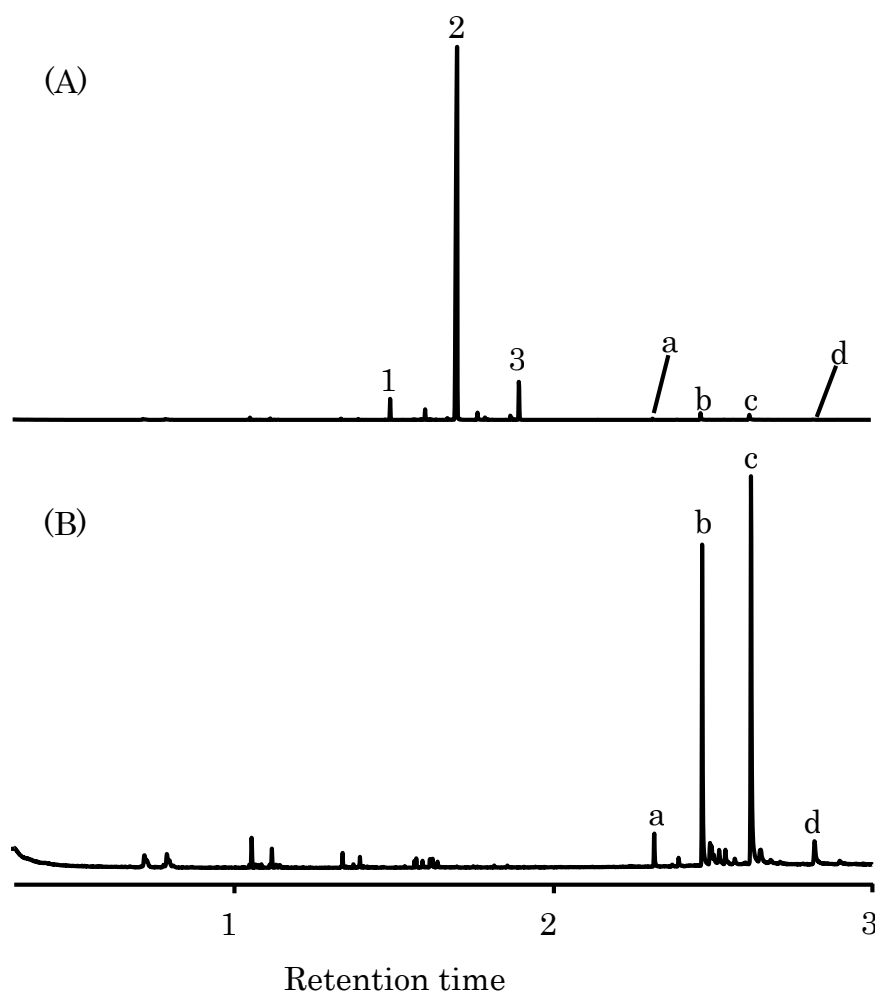


Fig. 3.2. Gas chromatographic comparison of crude extracts from (A) an adult male, and (B) an adult female; four peaks (a, b, c and d, identified as C₂₅, C₂₇, C₂₉, and C₃₁, respectively) were found in both extracts, while three peaks (compounds 1, 2 and 3) were found to be male-specific components.

3.3.3. Purification and isolation of chemical compounds

Crude extracts from 100 adults were separated on an SiO₂ column into five fractions. Each fraction was subjected to GC-MS. The male-specific components (1–3) were detected only in the fraction eluted with 10% EtoAc in hexane. To assess pheromonal activity, all fractions were bioassayed using a method similar to that of the previous bioassay involving crude extracts, as

summarized in Table 3.3. There was a significant difference in the number of both female and male beetles that aggregated on the disk treated with 10% EtoAc in hexane fraction (Table 3.3).

Furthermore, the 10% EtoAc in hexane fraction elicited stronger aggregative behavior in adult *L. africanus* beetles than did the other fractions, as indicated by the AI values of the adult beetles, particularly females (female: $F= 96.08$, $df= 49$, $P < 0.0001$; male: $F= 11.77$, $df= 49$, $P < 0.0001$), as shown in Table 3.4.

Table 3.3. Aggregation of adult *L. africanus* to disks treated with some fraction of male crude extract indicated by percentage of beetles ($N= 20$; $n = 10$)

Fractions	Tested beetles	% responder beetles		Pvalue
		Treated	Control	
Fr.1	♀	6.83 ± 1.25	15.33 ± 1.53	0.031*
	♂	2.75 ± 0.80	7.83 ± 2.28	0.031*
Fr.2	♀	54.85 ± 2.61	5.30 ± 1.12	0.002*
	♂	31.90 ± 2.82	14.45 ± 2.68	0.006*
Fr.3	♀	17.60 ± 2.66	11.10 ± 2.58	0.125
	♂	17.20 ± 3.27	6.30 ± 1.35	0.063
Fr.4	♀	13.10 ± 1.56	9.30 ± 1.91	0.188
	♂	3.90 ± 1.26	10.40 ± 2.85	0.188
Fr.5	♀	8.30 ± 1.62	10.20 ± 2.33	0.063
	♂	4.80 ± 0.64	5.80 ± 2.26	1.000

Notes: Level of significant differences between numbers of beetles on disk are shown by asterisks (Matched pairs test). Fr.1: hexane; Fr.2: 10% EtoAc in hexane; Fr.3: 20% EtoAc in hexane; Fr.4: 50% EtoAc in hexane; Fr.5: EtoAc.

Table 3.4. Response of adult *L. africanus* to disks treated with fractions of male crude extract (50 μ L = 2.5 beetles equivalent), presented by aggregation index value ($N = 20$; $n = 10$)

Tested beetles	Fractions				
	Fr.1	Fr.2	Fr.3	Fr.4	Fr.5
♀	(-)0.04 \pm 0.02 ^{b(ns)}	0.50 \pm 0.03 ^{a(*)}	0.07 \pm 0.02 ^{b(ns)}	0.02 \pm 0.01 ^{b(*)}	0.03 \pm 0.02 ^{b(ns)}
♂	(-)0.07 \pm 0.02 ^{c(ns)}	0.18 \pm 0.04 ^{a(*)}	0.10 \pm 0.04 ^{ab(ns)}	(-)0.04 \pm 0.02 ^{bc(*)}	(-)0.02 \pm 0.03 ^{bc(ns)}

Notes: Aggregation index value (mean \pm SEM), which indicates significant difference ($p < 0.01$) by different letters within treatments (for females and males) according to Tukey's HSD test. Letters in parentheses refer to the comparison between female and male beetles in the same fraction (Student's *t* test).

*: Significant; ns: not significant. Fr.1: hexane; Fr.2: 10% EtoAc in hexane; Fr.3: 20% EtoAc in hexane; Fr.4: 50% EtoAc in hexane; Fr.5: EtoAc.

3.3.4. Identification and syntheses of the chemical compounds

Compound **2** was the most abundant product in the 10% EtoAc in hexane fraction, as indicated by M^+ ion at m/z 270 and the base ion at m/z 183 with diagnostic ion at m/z 201 (Fig. 3.3.B). The latter ion was the second most intense ion, indicative of a fragment derived from a C_{12} -fatty acid moiety. The base ion recorded at m/z 183 (Fig. 3.4.D) was the dehydrated m/z 201 ion. The third most intense ion at m/z 70 (57%) was found to be derived from a C_5 -alcohol moiety (Fig. 3.4.C). The mass spectrum of compound **2** resembled that of *n*-pentyl dodecanoate (*n*-pentyl laurate), although the GC t_R of compound **2** (16.95 min) was shorter than that of *n*-pentyl dodecanoate (17.52 min) [55]. These findings suggested an isomeric relationship between the two compounds that compound **2** was a dodecanoate of a branched alcohol. In addition, a fatty ester containing an *n*-pentyl alcohol moiety showed a

fragment ion of m/z 70 as the base ion (compound **4** (*n*-pentyl dodecanoate) and **6** (*n*-pentyl isotridecanoate)) [55]. Four monobranched candidates were prepared as candidate structures for the comparison of the GC t_R to that of compound **2**. 2-pentyl dodecanoate (2-PD), 2-methylbutyl dodecanoate (2-MBD), and 3-methylbutyl dodecanoate (3-MBD) were synthesized using the same procedure employed in the synthesis of 3-pentyl dodecanoate **2** via a reaction between the corresponding alcohols and dodecanoyl chloride. Synthetic 3-pentyl dodecanoate yielded a GC t_R and mass spectrum that were identical to those of natural product **2**. Compound **2** was identified as 3-pentyl dodecanoate (Fig. 3.5.B).

The diagnostic ion of compound **1** recorded at m/z 201 was identified as that of a C₁₂ fatty acid, as mentioned above (Fig. 3.3.A). The ester containing a 2-propyl alcohol moiety showed fragment ions of m/z 43 (Fig. 3.4.A) and m/z 60 (Fig. 3.4.B) as the base and diagnostic ions, respectively. Accordingly, compound **1**, which showed an M⁺ ion at m/z 242, was identified as 2-propyl dodecanoate **1** (14.92 min). Authentic compound **1** gave the same GC t_R and mass spectrum as natural product **1**. The fragment ion at m/z 229 of compound **3** was comparable to that of a C₁₄-fatty acid moiety (Fig. 3.3.C). The base ion recorded at m/z 211 (Fig. 3.4.E) was the dehydrated m/z 229 ion. The diagnostic ion at m/z 70 was similar to that of compound **2**, which suggested that **3** was an ester containing a 3-pentyl alcohol moiety. The structure of **3** was thus assigned as that of 3-pentyl tetradecanoate. Authentic compound **3** gave the same GC t_R and mass spectrum as did natural product **3**. Compound **1** was accordingly identified as 2-propyl dodecanoate, and compound **3** as 3-pentyl tetradecanoate (Fig. 3.5.A and C).

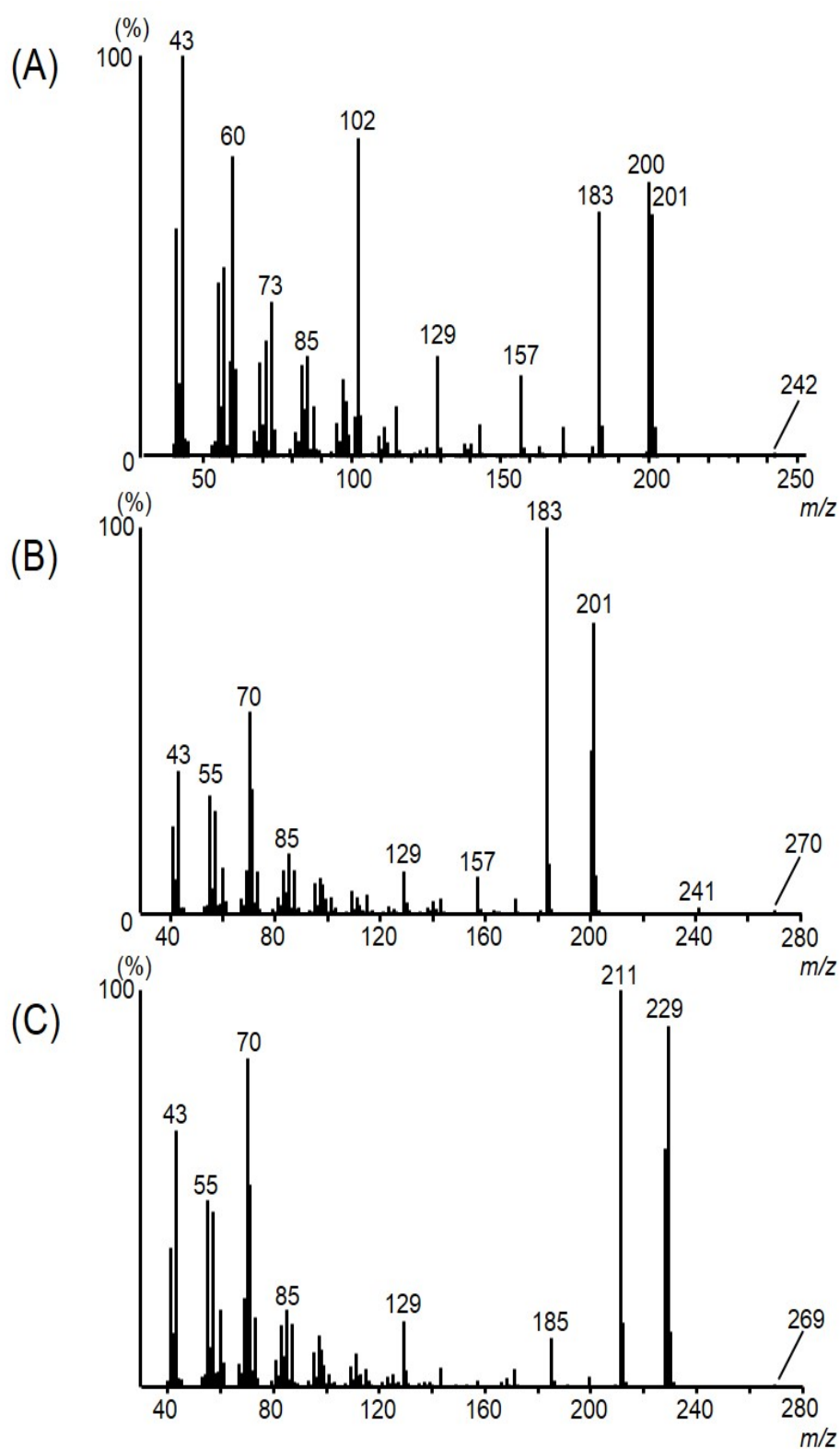


Fig. 3.3. Mass spectra of three esters: (A) **1**; (B) **2**; and (C) **3**.

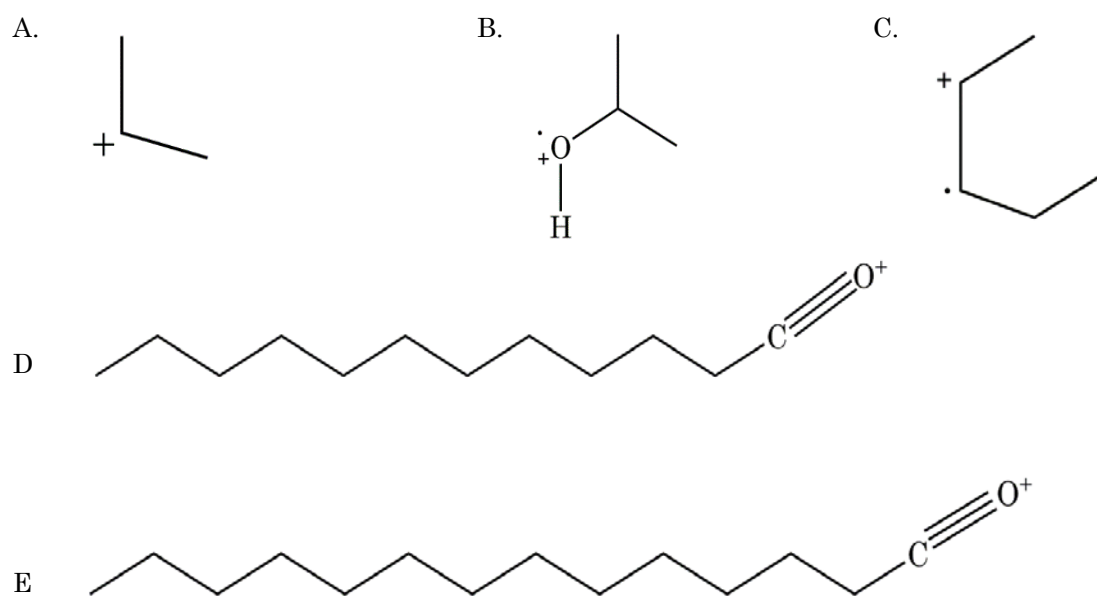


Fig. 3.4. The fragment ions at (A) m/z 43; (B) m/z 60; (C) m/z 70; (D) m/z 183 and (E) m/z 211

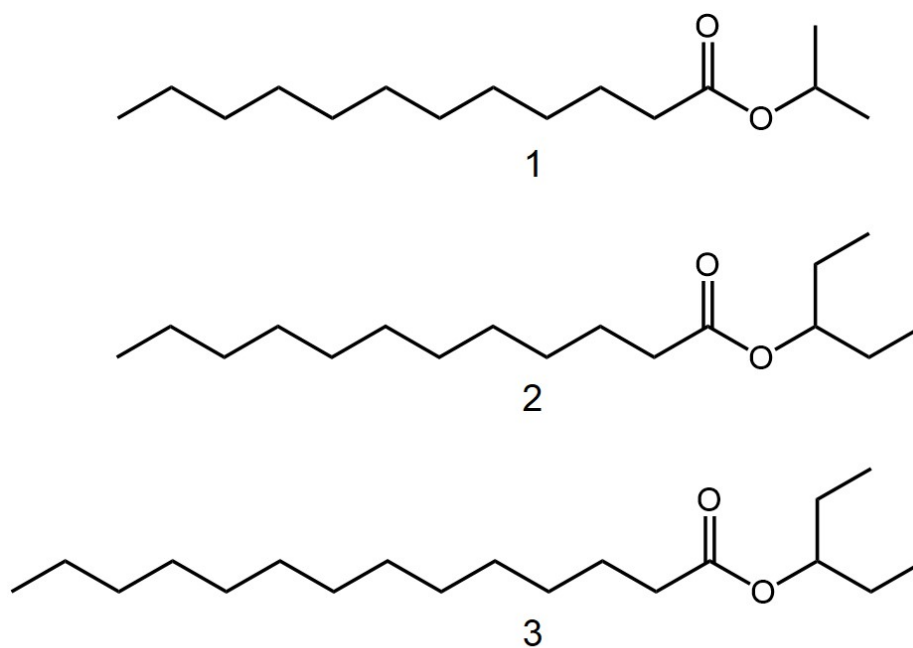


Fig. 3.5. Chemical structures of compounds (A) 1; (B) 2; and (C) 3.

On the basis of GC–MS analysis, three esters were identified in male crude extract. The esters were synthesized to confirm their activities on adult *L. africanus*. Dodecanoyl chloride (1.0 g, 4.57 mmol) in diethyl ether (5 ml) was added dropwise to a solution of 3-pentanol (0.41 g, 4.66 mmol) and pyridine (0.37 g, 4.66 mmol) dissolved in diethyl ether (10 ml) at 0 °C. The mixture was stirred at room temperature for 60 min. After filtration, the eluate was concentrated and then applied to an SiO₂ column. Elution with 10% EtOAc in hexane afforded 1.17 g (4.34 mmol, 95%) of 3-pentyl dodecanoate **2** as a colorless oil. GC–MS t_R : 16.95 min. ¹H NMR (CDCl₃, δ ppm): 0.88 (t, 9H, J = 6.6 Hz, CH₃), 1.21–1.34 (m, 16H, CH₂), 1.52–1.64 (m, 6H, CH₂), 2.31 (t, J = 7.2 Hz, 2H, CH₂CH₂CO), 4.76 (tt, J = 6.8, 5.6 Hz, 1H, OCH(CH₂)₂); ¹³C NMR (CDCl₃, δ ppm): 173.8, 76.4, 34.7, 31.9, 29.6 (\times 2), 29.5, 29.3, 29.3, 29.2, 26.5 (\times 2), 25.2, 22.7, 14.1, 9.6 (\times 2) (Fig. S2A and S2B, Supporting information).

The remaining 2 esters, 2-propyl dodecanoate **1** and 3-pentyl tetradecanoate **3**, were obtained using the same procedure employed in the synthesis of 2-propyl dodecanoate **1**, via reaction between the corresponding acyl chlorides and alcohols. 2-propyl dodecanoate **1**: GC–MS t_R : 14.92 min. ¹H NMR (CDCl₃, δ ppm): 0.88 (t, 3H, J = 6.6 Hz, CH₂CH₃), 1.25 (d, 6H, J = 4.0 Hz, CH(CH₃)₂), 1.21–1.33 (m, 16H, CH₂), 1.61 (m, 2H, CH₂CH₂CO), 2.25 (t, J = 7.6 Hz, 2H, CH₂CH₂CO), 5.00 (septet, J = 6.4 Hz, 1H, OCH(CH₃)₂); ¹³C NMR (CDCl₃, δ ppm): 173.5, 67.3, 34.8, 31.9, 29.6 (\times 2), 29.5, 29.3, 29.3, 29.1, 25.1, 24.7, 22.7, 21.9 (\times 2) (Fig. S1A and S1B, Supporting information). 3-pentyl tetradecanoate **3**: GC–MS t_R : 18.95 min. ¹H NMR (CDCl₃, δ ppm): 0.88 (t, 9H, J = 6.6 Hz, CH₃), 1.21–1.33 (m, 20H, CH₂), 1.52–1.64 (m, 6H, CH₂), 2.29 (t, J

= 7.6 Hz, 2H, CH₂CH₂CO), 4.76 (tt, $J = 6.8, 5.6$ Hz, 1H, OCH(CH)₂); ¹³C NMR (CDCl₃, δ ppm): 173.8, 76.3, 34.7, 31.9, 29.7, 29.6 ($\times 2$), 29.6, 29.5, 29.3, 29.3, 29.2, 26.5 ($\times 2$), 25.2, 22.7, 14.1, 9.6 ($\times 2$) (Fig. S3A and S3B, Supporting information).

3.3.5. Quantitative determination of the three compounds

Male beetles contained 15.1 ± 4.9 ng of 2-propyl dodecanoate **1**, 323.9 ± 121.2 ng of 3-pentyl dodecanoate **2**, and 20.1 ± 13.1 ng 3-pentyl tetradecanoate **3** on average, whereas females contained no trace of the corresponding compounds.

3.4. Discussion

The male crude extract *L. africanus* induced the aggregative behaviour for both males and females within a short time after releasing into the Petri dish. Furthermore, the beetles frequently stayed on treated paper disc throughout five minutes observation. The rapid recruitment of beetles to male crude extract and their behaviour on treated disc through bioassay would suggest that the compounds are attractants. In contrast, the female crude extract produced insignificant responses in both male and female beetles. A similar result was reported in closely related Bostrichidae (Coleoptera) genera. Males of the lesser grain borer (*Rhyzopertha dominica*) [61] and larger grain borer (*Prostephanus truncatus*) [62] produced attractants which acted as a population-aggregating pheromones that attracted conspecifics females and males. These two stored-product beetles released the aggregation

pheromone that aid in the location of food sources and suitable breeding sites [63,64].

For long-lived insects (>1 month), the aggregation pheromone plays a significant role in signalling the presence of both food sources and mates [16]. The lyctines, including *L. africanus*, are considered to be long-live adult insects and have been reported to lived three to six weeks in previous studies [23,25,32] and up to 13 weeks in our study (data not shown).

Using GC-MS, three major compounds were identified in female and male crude extracts. Hydrocarbons (C₂₅, C₂₇, C₂₉ and C₃₁) were identified in the female and male crude extract, while esters (isopropyl dodecanoate (1), 3-pentyl dodecanoate (2) and 3-pentyl tetradecanoate (3)) were recognized as specific compounds in the male crude extract.

In general, hydrocarbon comprise a significant portion of the cuticular lipids of insects. The compound could prevent desiccation and are also important in chemical communication in insect [65]. Some insects utilized hydrocarbons as sex attractants which released by females [66-68]. The male rustic borer, *Xylotrechus colonus* F. (Coleoptera: Cerambycidae) recognizes females by distinguishing the female`s cuticular hydrocarbon containing *n*-pentacosane, 9-methylpentacosane, and 3-methylpentacosane (identified as female-specific compounds) [69]. On the basis of our results, hydrocarbons were not involved in chemical communication among this species.

Moreover, some esters were reported as insect pheromone in *Dermestes* sp. [70]. In addition, some esters were identified as major component in the wings of male butterfly, *Colias philodice* [71]. In that study, esters were

reported as important cues in species recognition. The closely related species, *R. dominica* and *P. truncates*, also produced esters as pheromones [61,72,62].

Separated fractions on a SiO₂ column showed different behavior responses of adult *L. africanus*. The 10% EtoAc in hexane fraction induced the greatest aggregation behavior, whereas the other fractions tested showed non-aggregation responses. The active fraction, also containing three esters, was then called the ester fraction. These results confirmed the function of purified ester in the aggregation behavior of adult *L. africanus*. In our study, ester compounds were likely attract both male and female adult *L. africanus* to aggregate in a group. Thus, esters were then recognized triggering aggregation behavior in *L. africanus*.

Chemical analyses revealed that compound **2** was found exclusively in male beetles as a major component. This compound was detected in large amount (323 ng/beetle), followed by compound **3** (20 ng/beetle) and compound **1** (15 ng/beetle). These amounts may be substantially larger than the actual amounts released by beetles. A headspace analysis may be useful for detecting actual volatile emissions. However, none of the esters were detected on male *L. africanus* using solid-phase micro-extraction sampling (N. Shimizu, personal communication). The esters could not be traced using this method, because of their low volatility. Another study on the storage mite, *Chortoglyphus arcuatus*, also reported that solvent extract and headspace sampling detected different chemical compounds [56].

Compound **1** is known as male-specific component of the black larder beetle *Dermestes haemorrhoidali* that acts as an aggregation pheromone [70,54], and is also a major component of the male abdominal exocrine glands

of the black larder beetle *Dermestes ater* [73]. This same compound also acts as a minor component of the labial glands of the stingless bee *Trigona corvine* [73]. Compound **3** has not been previously reported.

The 3-pentyl dodecanoate (compound **2**) was recognized as the major active compound of the aggregation pheromone in *L. africanus*. In view of the large amounts produced by males, it is reasonable to assume that this pheromone plays an important role in the ecology of *L. africanus*. This is the first report of 3-pentyl dodecanoate (**2**) as a natural product.

3.5. Conclusions

Our study is the first record of pheromone identification in the *Lyctus* beetle. To date, pheromone production in the Bostrichidae family has been found only in two stored-product pests, the lesser and larger grain borers [61,62]. In this study, esters were detected as aggregation pheromone in *L. africanus* beetles, which attracted both male and female beetles. 3-pentyl dodecanoate (compound **2**) was recognized as the major active compound of the aggregation pheromone in *L. africanus*, along with other two minor compounds. Hopefully, this study findings will lead to additional research in the development of adult beetle monitors and control strategies for Bostrichidae, including *Lyctus*.

In next chapter, synthetic compound of three esters were evaluated regarding their aggregation activity against *L. africanus* beetle.

3.6. Summary

This chapter discussed the aggregation behavior of *L. africanus*. The behavior was investigated using a chemical approach through comprehensive screening of the potential compound produced by *L. africanus*.

The whole body extractions using hexane solvent on newly emerged beetles were performed. The biological activity of crude extract of *L. africanus* beetles was conducted by dual-choice bioassay. Then, it was followed by chemical analysis, isolation, identification and syntheses of the chemical compounds.

The results revealed three esters as an aggregation pheromone produced by male *L. africanus* beetle. The esters were recognized as a major compound (3-pentyl dodecanoate) and two minor compounds (2-propyl dodecanoate and 3-pentyl tetradecanoate).

Chapter 4. Aggregation activity of synthetic esters on *Lyctus africanus*

4.1. Introduction

Male of *L. africanus* beetles have been recognized triggering both female and male *L. africanus* beetles forming a group around the treated paper disk, as described in Chapter 3. Chemical analysis using GC-MS revealed the specific compounds produced by male *L. africanus*, which are then suggested as aggregation pheromones. Compounds of the male crude extract *L. africanus* were known to attract both female and male beetles in laboratory testing. Further steps on purification and isolation of the compounds using silica gel chromatograph revealed three esters of 2, propyl dodecanoate, 3-pentyl dodecanoate and 3-pentyl tetradecanoate.

The aggregation activity of crude extract (natural compound) was then compared with the responses to synthesized compound. The synthetic of three esters were then provided for further study. The aggregation activity of synthetic esters beetles were also conducted to verify the role of synthetic compounds in aggregation behavior of *L. africanus* beetles.

In this chapter, the aggregation activities of synthetic esters were examined in either single or blended compounds against *L. africanus* beetles.

4.2. Materials and Methods

The biological activity of synthetic compounds were tested in a laboratory dual-choice bioassay to show the aggregation behavior of *L. africanus*. First, the activity of a single compound was determined, then followed by the

blended compound activity to verify whether synergism occurred due to mixed chemical combination.

4.2.1. Biological activity of single ester

Adult male and female beetles were then exposed to the identified esters, single compound **1-3**, to assay the function of each compound in the chemical ecology of the powder-post beetle *L. africanus*. The bioassays were performed in closed Petri dishes using a dual-choice method similar to that of preceding bioassays. Synthetic compounds **1-3** were prepared in serial doses of 2, 20, 200, 400 and 800 ng/disk, then subjected to both male and female beetles.

The result of single compound bioassays determined the further step of creating the blended compounds. The blended compound formulations were created on the basis of optimum response of *L. africanus* to single ester.

4.2.2. Biological activity of ester blends

In order to verify the activity of synthetic esters, ester activity was evaluated by combining each ester into a blend. Firstly, three esters were combined into a blend in order to obtain an optimum ratio of each compound.

Two component blends were created, on the basis of the ratio of each single compound: a natural ratio (blend A), and a modified ratio (called blend B). The two blends were prepared by mixing synthetic compounds **1, 2, and 3** in the mass ratios 20:300:20 (blend A) or 300:300:300 (blend B). The natural ratio matched the actual proportions of the ester compounds detected in an *L. africanus* male beetle, whereas the modified ratio was an equal ratio of each compound. The activities of the blends were tested using a method similar to

that of the previous bioassay.

To examine the precise blended combination of esters, a two-blended combination was also performed whether the *L. africanus* highly attracted to combination of two- or three-esters. The two-ester combinations were the blend of compounds **1+2**, **1+3**, and also **2+3**. The ratio of quantitative amount of each compound in a blend was based on the result of three-ester combination test. A dual-choice method was also performed.

4.2.3. Statistical analysis

Ten replicates were made for each test. The counts of beetles preferring either treated or untreated (control) paper disks were scored after five minutes of observation and analyzed for significant differences. To determine the responses of beetles with regard to the extract, an aggregation index (AI) value was calculated (described in Chapter 3). The index values of the responses were subjected to one-way of analysis of variance, followed by Tukey's honest significant differences test (HSD) test ($\alpha = 0.05$), or Student's *t*-test (JMP, version 9, SAS Ins.).

4.3. Results

4.3.1. Activity of single compound

The biological activity of each single compound against adult *L. africanus* beetles is illustrated in Table 4.1. The adult male and female beetles showed similar responses to all compounds with different doses, excluding compound **2** at the highest dose of 800 ng/disk. Both male and female beetles were likely to aggregate to the high dose of compound **2**, however the AI values were not

high (0.23 ± 0.03 and 0.19 ± 0.03 for female and male beetles, respectively). Generally, female beetles showed the significant preference on the treated paper discs rather than the control paper discs (Table 4.2.).

Table 4.1. Response of adult *L. africanus* to treated disks with synthetic single compound **1**, **2** and **3** by aggregation index value ($N=20$; $n=10$)

Tested beetles	Doses (ng/disk)	Compound		
		1	2	3
♀	2	$0.05 \pm 0.04^{a(a)}$	$0.07 \pm 0.02^{a(b)}$	$(-)0.00 \pm 0.03^{a(a)}$
	20	$0.08 \pm 0.05^{a(a)}$	$0.14 \pm 0.02^{a(ab)}$	$0.04 \pm 0.05^{a(a)}$
	200	$0.09 \pm 0.04^{a(a)}$	$0.17 \pm 0.04^{a(ab)}$	$0.09 \pm 0.04^{a(a)}$
	400	$0.08 \pm 0.03^{a(a)}$	$0.17 \pm 0.03^{a(ab)}$	$0.13 \pm 0.04^{a(a)}$
	800	$0.04 \pm 0.02^{b(a)}$	$0.23 \pm 0.03^{a(a)}$	$0.13 \pm 0.03^{ab(a)}$
Tested beetles	Doses (ng/disk)	1	2	3
		1	2	3
♂	2	$(-)0.02 \pm 0.03^{a(a)}$	$0.09 \pm 0.05^{a(a)}$	$(-)0.08 \pm 0.04^{a(a)}$
	20	$0.04 \pm 0.03^{a(a)}$	$0.05 \pm 0.03^{a(a)}$	$0.07 \pm 0.02^{a(a)}$
	200	$0.03 \pm 0.03^{a(a)}$	$0.05 \pm 0.03^{a(a)}$	$(-)0.04 \pm 0.04^{a(a)}$
	400	$(-)0.01 \pm 0.04^{a(a)}$	$0.14 \pm 0.03^{a(a)}$	$0.08 \pm 0.04^{a(a)}$
	800	$0.01 \pm 0.04^{b(a)}$	$0.19 \pm 0.03^{a(a)}$	$0.05 \pm 0.03^{ab(a)}$

Notes: Aggregation index value (mean \pm SEM), which indicates significant difference ($p < 0.01$) by different letters within compounds in the same dose (for females and males) according to Tukey's HSD test. Letters in parentheses refer to the comparison between doses on female or male beetles in particular compound (Tukey's HSD test). ns, not significant.

Table.4.2. Aggregation of adult *L. africanus* on disks treated with synthetic compound **1**, **2**, and **3**, indicated by percentage of beetles ($N= 20$; $n = 10$)

Compound	Tested beetles	Doses (ng/disk)	% responder beetles		Pvalue
			Treated	Control	
1	♀	2	18.90±3.09	13.50±2.27	0.232
		20	21.65±4.57	13.35±1.75	0.186
		200	19.85±2.56	11.10±2.16	0.018*
		400	21.90±3.07	14.35±1.66	0.027*
		800	13.50±1.10	9.50±2.02	0.074
	♂	2	14.60±2.61	16.20±1.48	0.537
		20	17.65±2.90	13.50±2.72	0.432
		200	14.20±1.85	11.75±1.97	0.264
		400	14.80±2.67	15.60±2.39	0.540
		800	12.15±3.03	11.15±2.03	0.748
2	♀	2	20.15±1.80	13.40±1.37	0.030*
		20	25.40±2.64	11.55±1.35	0.002*
		200	26.25±3.10	9.75±1.46	0.002*
		400	28.75±2.53	11.70±1.88	0.002*
		800	27.95±2.55	4.70±0.97	0.002*
	♂	2	25.05±4.00	16.30±1.73	0.047*
		20	14.70±3.53	9.80±1.88	0.201
		200	18.30±2.25	13.85±2.65	0.201
		400	24.15±2.98	10.30±1.67	0.006*
		800	24.3±2.26	5.35±1.40	0.002*
3	♀	2	16.80±3.15	15.95±2.06	0.902
		20	16.85±4.31	13.40±1.82	0.695
		200	21.00±4.12	12.05±2.32	0.084
		400	23.90±4.18	10.95±1.34	0.014*
		800	18.30±2.58	5.50±1.27	0.012*
	♂	2	10.30±2.25	20.70±2.32	0.045*
		20	16.95±1.96	10.15±2.24	0.022*
		200	12.30±2.97	16.50±3.26	0.361
		400	22.25±3.41	14.80±1.80	0.087
		800	13.65±2.25	8.65±2.11	0.098

Notes: Level of significance difference for number of beetles on disc, shown by asterisk symbols (Matched pairs test).

4.3.2. Activity of three-esters blend

Result from our bioassays suggest both female and male beetles aggregated more on treated than control disks (Table 4.3). The AI value (Table 4.4) showed that both female and male aggregation on paper discs treated with blend A was significantly higher than on those treated with blend B (Student *t*-test, $P < 0.05$).

Table 4.3. Response of adult *L. africanus* to treated disks with two types of blend, blend A and B, indicated by aggregation index value ($N = 20$; $n = 10$)

Tested beetles	Blend A	Blend B
♀	0.46 ± 0.042 ^{*(ns)}	0.24 ± 0.02 ^{*(ns)}
♂	0.34 ± 0.05 ^{*(ns)}	0.21 ± 0.03 ^{*(ns)}

Notes: Aggregation index value (mean ± SEM), with significant ($p < 0.01$, Student's *t* test) differences indicated by asterisks within the same sex, whereas letters in parentheses refer to comparison (by Tukey's HSD test) between female and male beetles. ns, not significant.

Table.4.4. Aggregation of adult *L. africanus* on disks treated with two component blends ($N = 20$; $n = 10$)

Treatment	Tested beetles	% responder beetles		<i>P</i> value
		Treated	Control	
Blend A	♀	48.80 ± 4.45	2.65 ± 0.82	0.002*
	♂	45.50 ± 3.43	12.00 ± 2.67	0.002*
Blend B	♀	27.00 ± 1.97	3.45 ± 1.10	0.002*
	♂	22.60 ± 3.47	1.40 ± 0.43	0.002*

Notes: Level of significant differences between numbers of beetles on disk are shown by asterisks (Matched pairs test).

4.3.3. Activity of two-esters blend

The modified ratio was formulated on the basis of the quantity of major compound (compound **2**) detected in a beetle. Based on the results of three-ester bioassay, the modified compound created based on the quantity of the major compound regarding its high impact on triggering *L. africanus* beetle forming a group.

The result of two-ester bioassays is presented in Fig. 4.1. Combination of **1+2** and **2+3** significantly attracted more female beetles than combination **1+3**, however showed similar responses on male beetles.

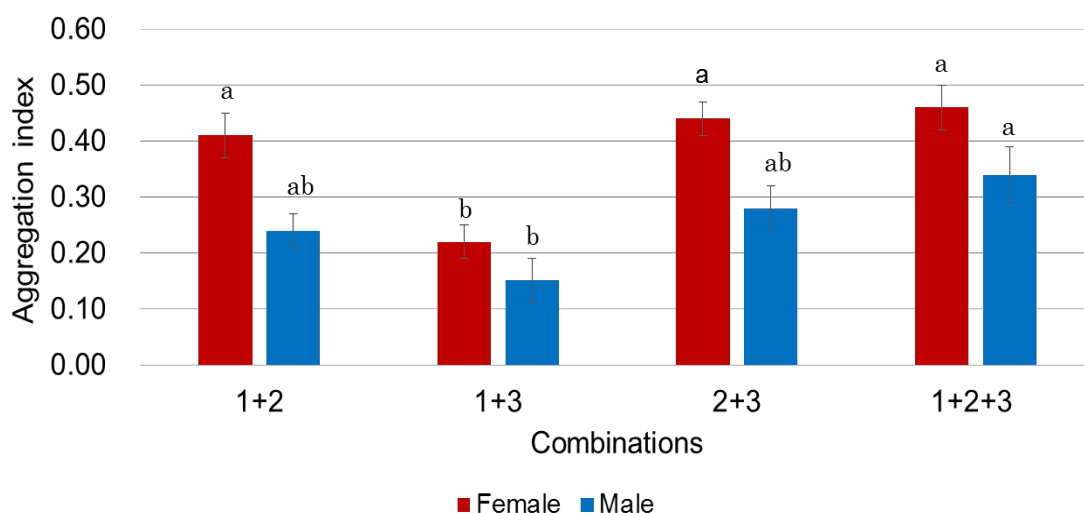


Fig. 4.1. Response of adult *L. africanus* against three types of dual combinations. Notes: Levels not connected by same letter are significantly different among female or male beetles (Tukey HSD test), while the letters in parentheses refer to the comparison of male and female beetles (Student's).

Furthermore, the activity of two-ester and three-ester combinations was evaluated, as presented in Table 4.5. As shown by the AI value, all combinations, excluding **1+3** combination, significantly attracted female beetles ($F = 9.31$, $df = 39$, $P = 0.0001$). Meanwhile, male beetles were highly

attracted by **1+2+3** combination, then followed by combinations **2+3**, **1+2**, and the lowest one was **1+3** combination ($F= 3.77$, $df= 39$, $P=0.0189$).

Table 4.5. The response of adult *L. africanus* to various combinations, as indicated by AI value ($N= 20$; $n = 10$)

Combinations	Tested beetles	
	♀	♂
1+2	0.41 ± 0.04 ^{a(*)}	0.24 ± 0.03 ^{ab(*)}
1+3	0.22 ± 0.03 ^{b(ns)}	0.15 ± 0.04 ^{b(ns)}
2+3	0.44 ± 0.03 ^{a(*)}	0.28 ± 0.04 ^{ab(*)}
1+2+3	0.46 ± 0.04 ^{a(ns)}	0.34 ± 0.05 ^{a(ns)}

Notes: Aggregation index value (mean ± SEM), with significant ($p<0.05$) differences indicated with different letters in the same according to Tukey's HSD test, whereas the asterisks in parentheses refer to the comparison between female and male beetles (Student' *t*-test); ns, not significant.

4.4. Discussion

A single synthetic compound **2** was found to induce the aggregative behavior of both female and male beetles at a high dose of 800 ng/disk, higher than its natural concentration. However, the responses elicited by the single synthetic compound **2** were not high compared with those by the purified natural compound. It is likely that the activity of the single compound **2** was not sufficient to induce the aggregation behavior of *L. africanus*. The other single minor compounds **1** and **3** seemed to have no effect on the beetle's responses.

Furthermore, there was a synergistic effect among the three synthetic

esters, compounds **1–3**, which increased the preference of both male and female beetles for only the natural blend. Some insects are aggregated by blends of pheromones consisting of two or more active compounds [74,70,75,76]. Our results revealed the synergism of the three synthetic esters in natural blend increasing aggregation. Meanwhile, the modified ratio was likely having extremely high concentration to attract the adult *L. africanus* beetles. In some insect species, it was reported that the increased released of specific chemical compounds or pheromones can also inhibit aggregation [21]. Accordingly, the natural blend was then used for further study.

Furthermore, bioassays on two-ester combinations were conducted to examine whether the aggregation pheromone contain two or three compounds in aggregation behavior of *L. africanus*. The two-ester combinations of **1+2** and **2+3** attracted more beetles of both sexes. Both combinations comprised the major compound **2** in the blend. It seems the compound **2** is a major active compound which can attract more adult *L. africanus* if being combined with other minor compounds. However, the females were more responsive than the male beetles.

Comparison of all combinations of two- and three-ester indicate that only the three-ester combination induced aggregation behavior of both female and male *L. africanus* beetles. These responses were similar with the natural compound of male crude extract which attracted both female and male beetles. It is suggested that the three compounds, **1**, **2** and **3**, are the component of aggregation pheromone of *L. africanus* beetles. For further study, three-ester combination are applied for more experiments.

4.5. Conclusions

The responses elicited by the single synthetic compound, such as compound **2** were not high compared with those by the purified natural compound. It is likely that the activity of the single compound was not sufficient to induce the aggregation behavior of *L. africanus*. The other single minor compounds **1** and **3** seemed to have no effect on the beetle's responses. Then, synergistic effect was found among the three synthetic esters, compounds **1–3**, which increased the preference of both male and female beetles for only the natural blend. Comparison of all combinations of two- and three-ester indicate the three compounds, **1**, **2** and **3**, are the component of aggregation pheromone of *L. africanus* beetles.

4.6. Summary

In order to justify the significant role of synthetic compounds to *L. africanus*, the aggregation activity of either single or blended esters was discussed in this chapter. The bioassays were conducted in laboratory by dual-choice test against *L. africanus* beetles.

The results indicated that the single compound was not sufficient to induce the aggregation behavior of *L. africanus*. Furthermore, the natural blended compound increased the aggregation responses of *L. africanus*. There was a synergistic effect was found among the three synthetic esters, compounds **1–3**.

Chapter 5. Aggregation response of *Lyctus africanus* in a semi-field condition with natural blend of esters.

5.1. Introduction

Behavioral studies have played important roles in isolating, identifying, and synthesizing insect pheromones. It has been reported in the previous chapters, Chapter 3 and 4, which the male *L. africanus* produces esters as aggregation pheromones which attract both female and male beetles in laboratory scale bioassays. Moreover, the synergistic effect occurs in a blend compound of three-ester combination with natural ratio.

In order to initiate a pheromone-based monitoring program, it is necessary to conduct several semi-field tests before being applied in the field. In this chapter, the optimum dose of three-ester blend was determined using dual-choice test before performing test in a wind tunnel. Wind tunnel is a tool to test the flight response to semiochemicals [77], has been used widely in insect pheromone studies [78,79]. The behavioral responses of adult *L. africanus* beetles under natural condition could be predicted in a wind tunnel. Hence, the responses of *L. africanus* beetles against natural blend of synthetic esters in a wind tunnel are discussed in this chapter.

5.2. Materials and Methods

5.2.1. Insect sources

The adult *L. africanus* beetles were reared in wood-based artificial diets (see Chapter 1) inside a dark climatic chamber. For the bioassay, the newly emerged beetles were used.

5.2.2. Preparation of natural blend pheromone compounds with various doses

In the natural condition, total amounts of compounds **1**, **2** and **3** in a male beetle were identified as 15, 323, and 20 ng in averages. The blend of three synthetic compounds were prepared to determine the minimum dosage that elicited the maximum level of response from both female and male *L. africanus* beetles. The quantity of blend compound was multiplied on the basis of beetle equivalent from low to high doses in order to verify the optimum dose in aggregation behavior of *L. africanus*.

Quantitative information of each synthetic blended compound is described in Table 5.1.

Table. 5.1. Various doses of natural blend of esters for biological testing with adult *L. africanus*

Beetle- equivalent (BE)	Compound (ng)		
	1	2	3
1	20	300	20
5	100	1,500	100
10	200	3,000	200
20	400	6,000	400
30	600	9,000	600
50	1,000	1,5000	1,000
70	1,400	21,000	1,400
100	2,000	30,000	2,000

5.2.2. Bioassays of natural blend of esters using dual-choice test

To examine the responses of adults *L. africanus* beetles against various doses of natural blend of esters, a dual-choice bioassay (see Chapter 3) was conducted. The bioassay was conducted to screen the various doses of synthetic esters which enabled to trigger the greatest response of adult *L. africanus*. The responses were calculated into an aggregation index (AI). Detail information on AI values is shown in Chapter 3. The optimum dose inducing greatest aggregation response on *L. africanus* was applied for wind-tunnel bioassay.

5.2.3. Bioassays of natural blend of esters in a wind tunnel.

The response of adults *L. africanus* to synthetic esters was measured in a wind tunnel. Outside dimensions of the rectangular wind tunnel made by Plexiglas were 150 cm long and 50 x 50 cm square (Fig. 5.1). The tunnel was housed in a room with temperature ($25 \pm 2^\circ \text{C}$) and relative humidity ($50 \pm 20\%$) controls. Horizontal air flow with 35 cm/sec velocity was applied to create an atmosphere for an assay of pheromone's behavioral effect.

To run the bioassay, a 40 x 30 cm of a paper was placed at the bottom of the wind tunnel. The paper was used as arena for *L. africanus* to walk easily. The arena was divided into three areas of A, B, and C (Fig. 5.2). To run the bioassay, a small filter paper (5 cm in diameter, Whatman No.2, GE Healthcare, UK) was used as a source of either the synthetic esters (treatment) or hexane solvent (control), and then positioned in B area. Then, a group of 50 male or female beetles were released at releasing point (©) in A area (Fig. 5.2). The response of beetles on the arena was categorized into three

groups: non-responder, aroused, and attracted. The non-responders were the beetles which stayed on A area, while aroused ones were beetles staying on B area, and the attracted ones were the beetles that attached to 5 cm filter paper (C area). The observation was conducted for duration of 10 minutes. Five replications was applied for this study.

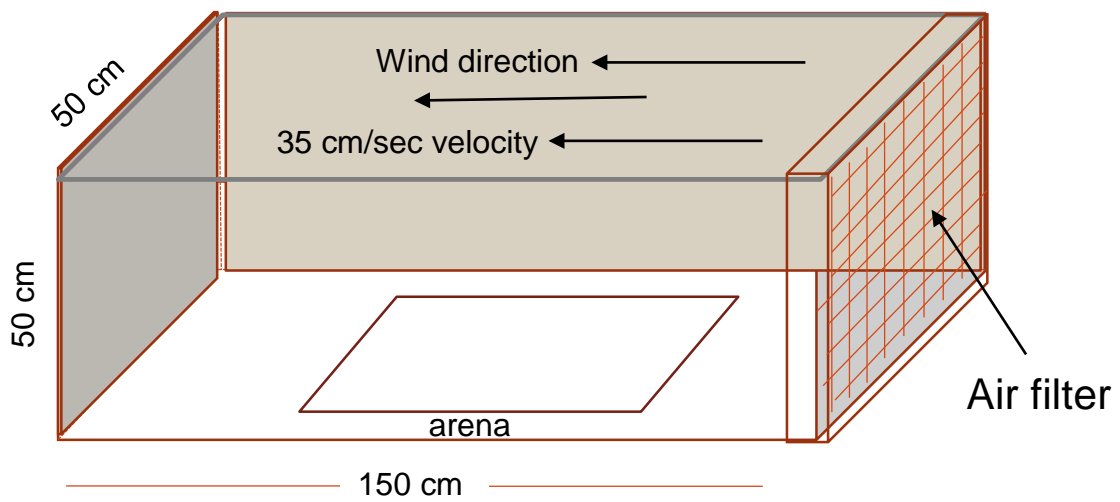


Fig. 5.1. A rectangular wind tunnel with push-type fan (35 cm/sec. of wind velocity).

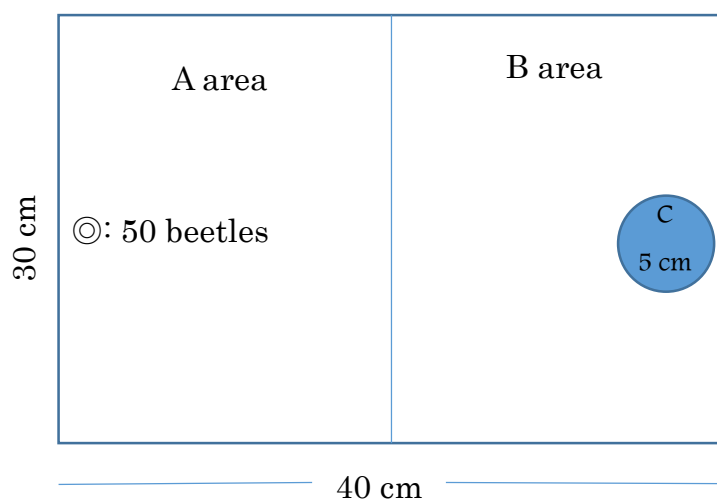


Figure 5.2. A rectangular paper for *L. africanus* arena in bioassay.

5.3. Results

5.3.1. Bioassays of synthetic blended chemicals using dual-choice test

The biological activity of each synthetic blend compound was illustrated in Fig. 5.3.

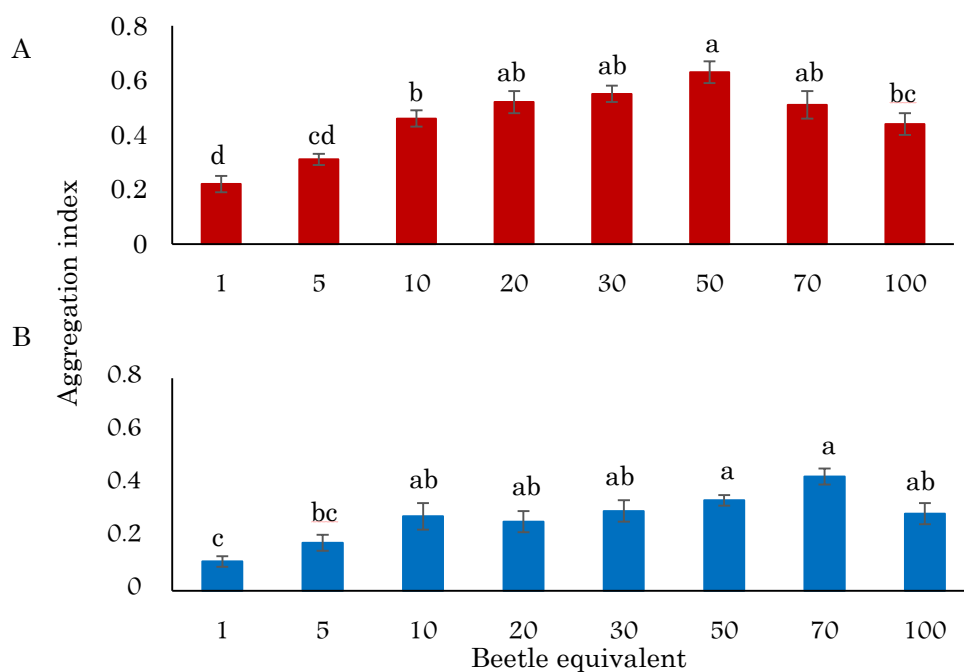


Figure 5.3. Response of female (A) or male (B) *L. africanus* exposed to various amounts of natural blend of synthetic esters.

Both female and male adults *L. africanus* showed a similar response. The responses of the aggregative behavior were increased as the increase of the doses of synthetic blend compound, then the responses started decreasing after achieving the highest responses at 50 BE for female, and at 50 or 70 BE for male beetles. The dose of 50 BE was then used in a wind tunnel bioassay.

5.3.2. Bioassays of natural blend of esters in a wind tunnel.

Responses of adults *L. africanus* exposed to blended compounds in a wind tunnel were illustrated in Table 5.2.

Table 5.2. Responses of adult *L. africanus* exposed to synthetic blend compounds

Tested beetles	Aroused beetles (%)			Attracted beetles (%)		
	Control	Treatment	<i>P</i> value ^[a]	Control	Treatment	<i>P</i> value ^[a]
♀	6 ± 2.61	48.8 ± 6.74	0.002*	0.0 ± 0.0	28 ± 2.97	0.007*
♂	12.8 ± 1.02	28.8 ± 5.64	0.046*	1.6 ± 0.98	8.0 ± 2.97	0.097

Notes: [a]: Level of significant different (Student's *t* test, *P* < 0.05).

A wind tunnel bioassays revealed that the synthetic blended compound of 50 BE significantly aroused and attracted female *L. africanus* beetles (Table 5.2). Almost half of the females (48.8%) showed an arousal response and 28% of the beetles touched the treated filter paper. On the other hand, the synthetic compound only evoked the arousal behavior on male *L. africanus* beetles. The male *L. africanus* beetles were not attracted to the synthetic blended compound.

5.4. Discussion

Male *L. africanus* was observed to produce aggregation pheromones that attracted both female and male beetles as described in Chapter 3. Some esters were identified as the active compound of the aggregation pheromones of *L.*

africanus. Then, the synthesized esters were provided for further studies. In this chapter, the optimum dose of the synthetic esters were evaluated. Based on the dual-choice bioassay in a closed Petri dish, it was determined that the responses of *L. africanus* changed as doses of the synthetic esters. Both female and male beetles oriented directly to the filter papers treated with higher doses of synthetic esters, however the attraction responses of both female and male *L. africanus* were decreased progressively at the highest dose used in this study (100 BE). These responses may have function to regulate density of colonization and limit the competition in the same species. Similar findings were reported that the increased dose of verbenone linearly reduced the number trap-catch of bark beetle, *Ips typographus* [80]. Another report mentioned that inhibition of male response by the higher concentrations of ipsenol, *cis*-verbenol, and ipsdienol encountered when the male-infested log was approached [81].

Through the wind tunnel bioassay, female and male *L. africanus* showed an arousal response to the treated filter paper with synthetic esters, indicating the significant function of synthetic esters to induce aggregation behavior of *L. africanus* in a wide area. Furthermore, it was observed that the male's responses were not high as female's. As described in the previous chapters, the responses of male were lower than those of female beetles in either crude extract or synthetic compounds. The aggregation pheromone might efficiently perform on female rather than male *L. africanus* beetles. Additional studies are necessary to strengthen the performance of synthetic compound for establishing the monitoring system of *L. africanus*.

5.5. Conclusions

The responses of *L. africanus* changed depending on the doses of the synthetic esters. Both female and male beetles oriented directly to the higher doses of synthetic esters, however the attraction response of both female and male *L. africanus* was decreased progressively at the highest dose used in this study. These responses may have function to regulate density of colonization and limit the competition in the same species.

The wind tunnel bioassay indicated that synthetic esters induce the aggregation behavior of *L. africanus*, particularly female, at a semi-field condition. The male's responses were not high as female's response suggesting the pheromone performed efficiently on female beetles.

5.6. Summary

A semi-field test was carried out in order to initiate a pheromone-based monitoring program in the future. In this chapter, the optimum dose of three-ester blend was determined using dual-choice test. The blend of three synthetic esters were prepared to determine the minimum dosage that elicited the maximum level of response from both female and male *L. africanus* beetles. Consequently, the optimum dose inducing greatest aggregation response on *L. africanus* was applied for wind-tunnel bioassay.

Results of dual-choice bioassays indicated that 50 BE induced the greatest response of aggregation behaviour of *L. africanus*. Furthermore, the wind tunnel bioassays revealed that both female and male beetles showed arousal response toward the natural blend of esters, however the male were less responsive than the female *L. africanus* beetles.

VI. Conclusions

In Chapter 1, the effect of artificial diet on fecundity of *L. africanus* was evaluated. An artificial diet should also provide the fitness of the *L. africanus*. The importance of host quality to assure availability of nutrient to generate more offspring, insect quality is also influenced by host quality during larva developmental stages. In this study, the females of *L. africanus* emerging from cellulose-based diets tend to be more fecund than those of wood-based diet. It was then suggested that *L. africanus* might utilize cellulose in their life cycles. However, the significant role of cellulose in life cycle of *L. africanus* is uncertain. Some previous studies reported that cellulose was not utilized by *Lyctus*. As a result, the wood-based diet was chosen for rearing the *L. africanus* for further study on the aggregation behavior of *Lyctus*.

Rearing Bostrichidae on an artificial diet may shorten the developmental growth and enlarge the number of progeny of these beetles. The life cycle of *L. africanus* was shorten in wood- and cellulose-based artificial diets. All three diets allowed a complete life cycle of *L. africanus*. However, the alpha-cellulose based diet could not support the growth of *L. africanus*. The wood-based (Diet 1) and cellulose-based diets (Diet 2) improved the growth of *L. brunneus*, thus these two diets could be used alternately. However, it was reported that malformations were found occasionally in the abdominal segmentation of *Lyctus* beetles that emerged from diets without sawdust. Thus, the utilization of cellulose-based diet (Diet 2) could be only utilized for mass culturing of *L. africanus* when sawdust or wood particles are not available.

This study is the first record of pheromone identification in the *Lyctus* beetle. To date, pheromone production in the Bostrichidae family has been found only in two stored-product pests, the lesser and larger grain borers. In this study, esters were detected as aggregation pheromone in *L. africanus* beetles, which attracted both male and female beetles. 3-pentyl dodecanoate (compound **2**) was recognized as the major active compound of the aggregation pheromone in *L. africanus*, along with other two minor compounds. Hopefully, these findings will lead to additional research in the development of adult beetle monitors and control strategies for Bostrichidae, including *Lyctus*.

In Chapter 4, synthetic compound of three esters were evaluated regarding their biological activity against *L. africanus* beetle. The responses elicited by the single synthetic compound, such as compound **2** were not high compared with those by the purified natural compound. It is likely that the activity of the single compound was not sufficient to induce the aggregation behavior of *L. africanus*. The other single minor compounds **1** and **3** seemed to have no effect on the beetle's responses. Then, synergistic effect was found among the three synthetic esters, compounds **1–3**, which increased the preference of both male and female beetles for only the natural blend. Comparison of all combinations of two- and three-ester indicate the three compounds, **1**, **2** and **3**, are the component of aggregation pheromone of *L. africanus* beetles.

The responses of *L. africanus* changed as the variation of the synthetic esters' doses. Both female and male beetles oriented directly to higher doses of synthetic esters, however the attraction response of both female and male *L. africanus* was reduced progressively at the highest dose used in this study.

These responses may function to regulate density of colonization and limit the competition in the same species.

The wind tunnel bioassay indicated that synthetic esters induce the aggregation behavior of *L. africanus*, particularly female, at a semi-field condition. The male's responses were not high as female's response suggesting the pheromone performed efficiently on female beetles.

VII. References

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VIII. Acknowledgement

Foremost, I would like to express my sincere gratitude to my supervisor Professor Tsuyoshi Yoshimura, Laboratory of Innovative Humanohabitability, Research Institute for Sustainable Humanosphere, Kyoto University, for the continuous support of my PhD and providing a good atmosphere for doing research. His guidance helped me in all of the time of research and writing this dissertation.

I would also like to thank Professor Yoshihisa Fujii from Laboratory of Wood Processing and Associate Professor Naoki Mori from Laboratory of Chemical Ecology, Kyoto University for their invaluable opinions, suggestions and cherished knowledge in reviewing and finalizing my dissertation.

My deeply gratitude goes to Associate Professor Nobuhiro Shimizu from Faculty of Bioenvironmental Science, Kyoto Gakuen University, who guide and provide me a new experience doing research in chemical ecology of insect. He shared me a basic knowledge in chemistry field.

My appreciation goes to Professor Masayuki Sakuma from Laboratory of Insect Physiology and Professor Toshiyuki Takano from Laboratory of Chemistry of Biomaterials, Kyoto University, for their kindness to share the knowledge and to let me use research facilities in their laboratory during the experiments. I also thank to Mr. Shin Tejima from Laboratory of Insect Physiology, Kyoto University who assisted me in wind tunnel research.

My sincere thanks also goes to Senior Lecturer Toshimitsu Hata and Assistant Professor Aya Yanagawa, Laboratory of Innovative Humanohabitability, Research Institute for Sustainable Humanosphere, Kyoto University, for the constructive comments and opinions to improve my

research. My deepest gratitude goes to Professor Emeritus Yuji Imamura and Associate Professor Kunio Tsunoda for their excellent encouragement and invaluable suggestions to my study.

Many special thanks to Dr. Kok-Boon Neoh and Dr. Lee-Jin Bong for their encouragement and insightful comments during my research. My sincere thanks also goes to Prof. Vernard Lewis from University of California, Berkeley, who share his knowledge in insect research and also improve my writing in manuscript preparation for publication.

And also, many thanks to Mr. Akio Adachi for his endless helpful assistance in preparing the experiments. I thank my fellow lab mates in Laboratory of Innovative Humano-habitability, Research Institute for Sustainable Humanosphere, Kyoto University: Mrs. Kaori Sunagawa, Ms. Izumi Fujimoto, Mrs. Kyoko Inoue, Mr. Baekyong Choi, Mr. Khoirul Himmi Setiawan, Ms. Ono Kazuko, Ms. Munadian, Mr. Didi Tarmadi, Mr. Ikhsan Guswenrivo, Mr. Sensho Honma and Mrs. Ai Tashiro for the stimulating discussions, for the technical assistance and for all of the fun we had in the last five years.

Many special thanks to my Japanese teacher, Mrs. Akemi Taniguchi for her great kindness during my stay in Japan. I am immensely in debt to my family, especially my dearest husband, lovely kids, adorable parents, sisters, brothers and other family member for all their sacrifices and for tirelessly giving encouragement and support throughout my life. Last but not the least, very special thanks and praises to God for the privilege of life and the wonderful journey within it.

IX. List of publications

Original articles

1. Titik Kartika and Tsuyoshi Yoshimura. 2013. Nutritional quality of diet and fecundity in *Lyctus africanus* (Lesne). *Procedia Environmental Sciences*, Vol. 17, Pages 97-104.
2. Titik Kartika and Tsuyoshi Yoshimura. 2015. Evaluation of wood and cellulosic materials as fillers in artificial diets for *Lyctus africanus* Lesne (Coleoptera: Bostrichidae). *Insects*, Vol. 6 (3), Pages 696-703.
3. Titik Kartika, Nobuhiro Shimizu, and Tsuyoshi Yoshimura. 2015. Identification of esters as novel aggregation pheromone component produced by the male powder post beetle, *Lyctus africanus* Lesne (Coleoptera: Lyctinae). *PlosOne* (accepted for publication in final editing).

X. Supporting information

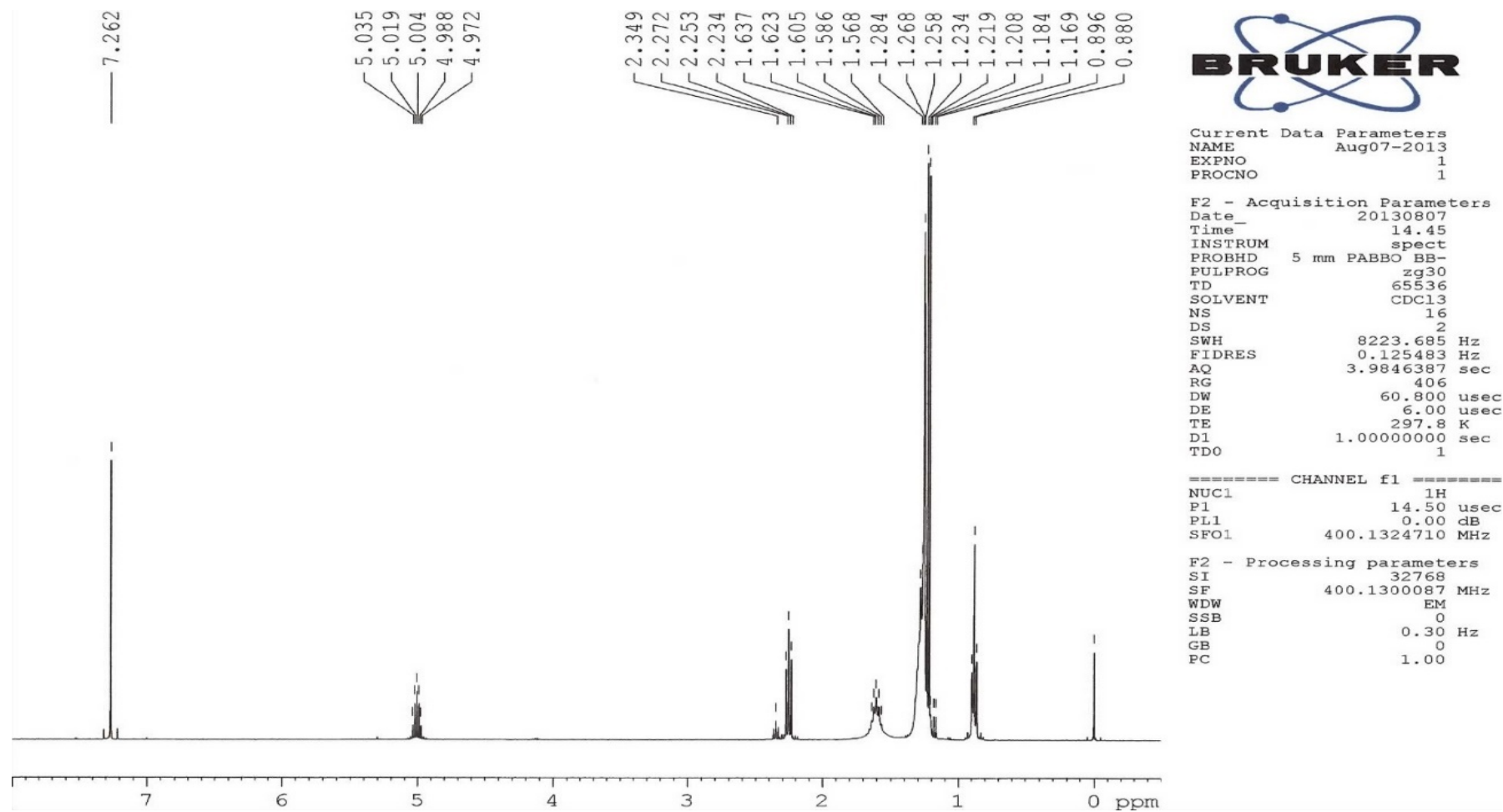


Fig. S1. The NMR spectrum of 2-propyl dodecanoate PMR

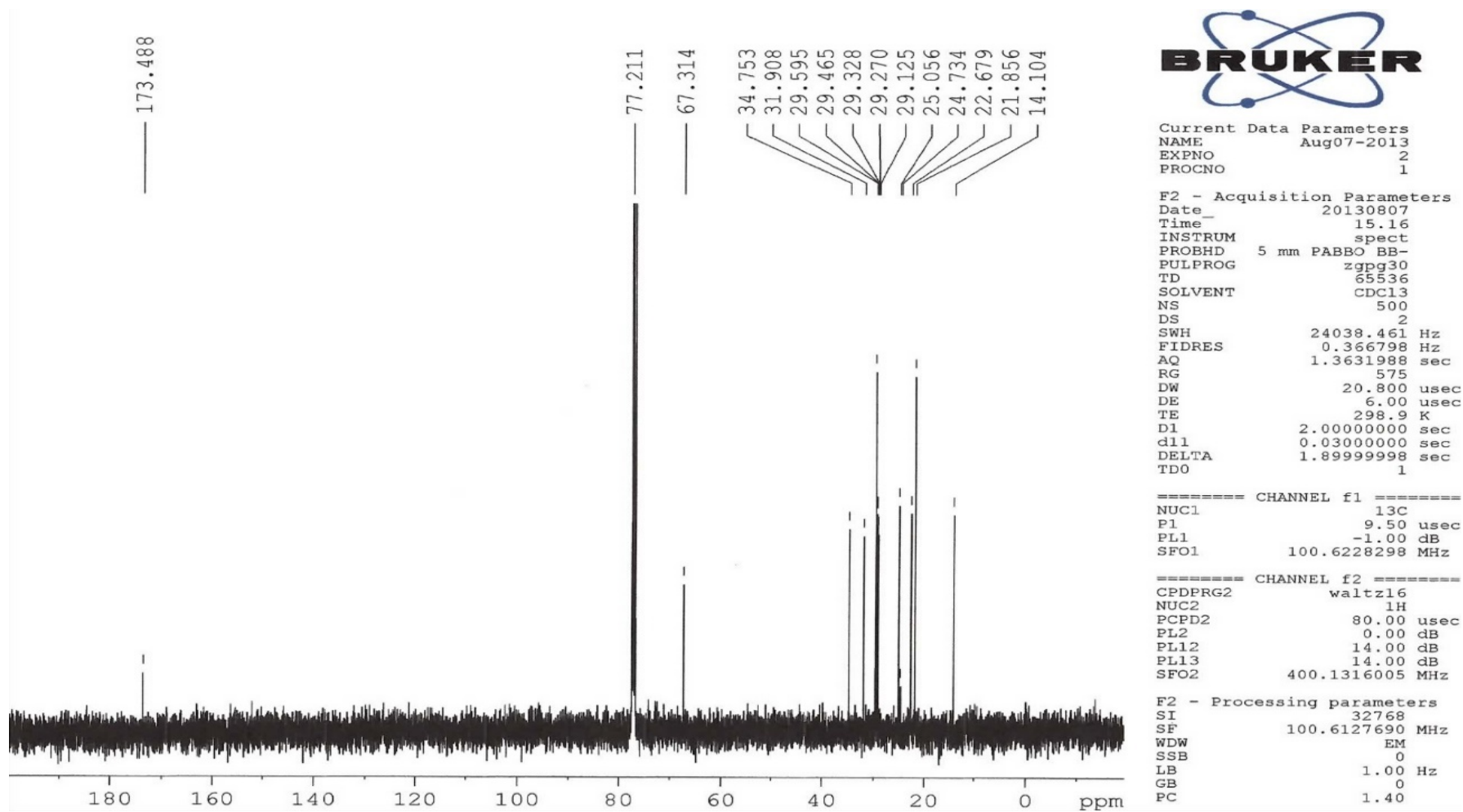
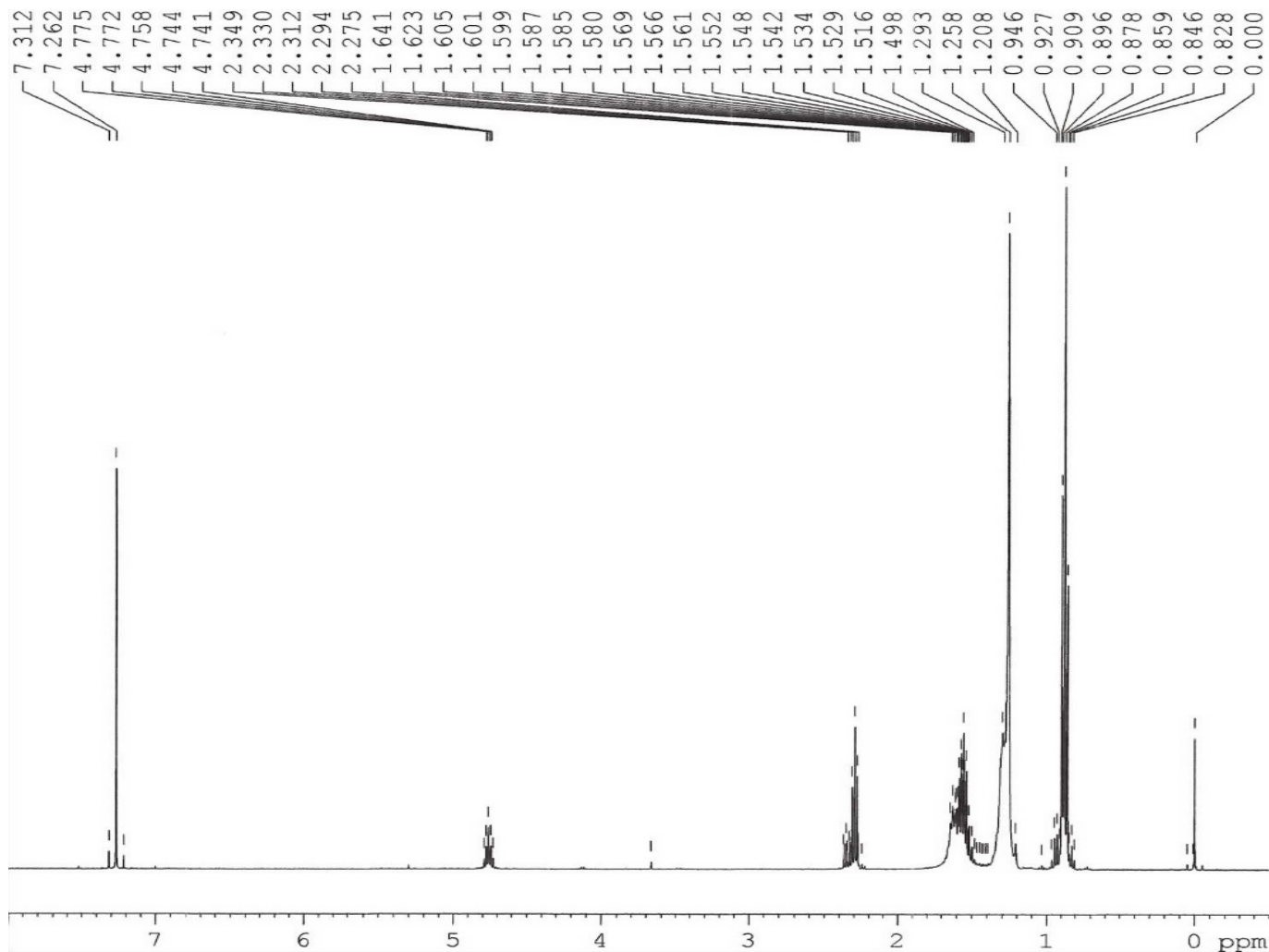


Fig. S2. The NMR spectrum of 2-propyl dodecanoate CMR



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F2 - Processing parameters
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Fig. S3. The NMR spectrum of 3-pentyl laurate PMR

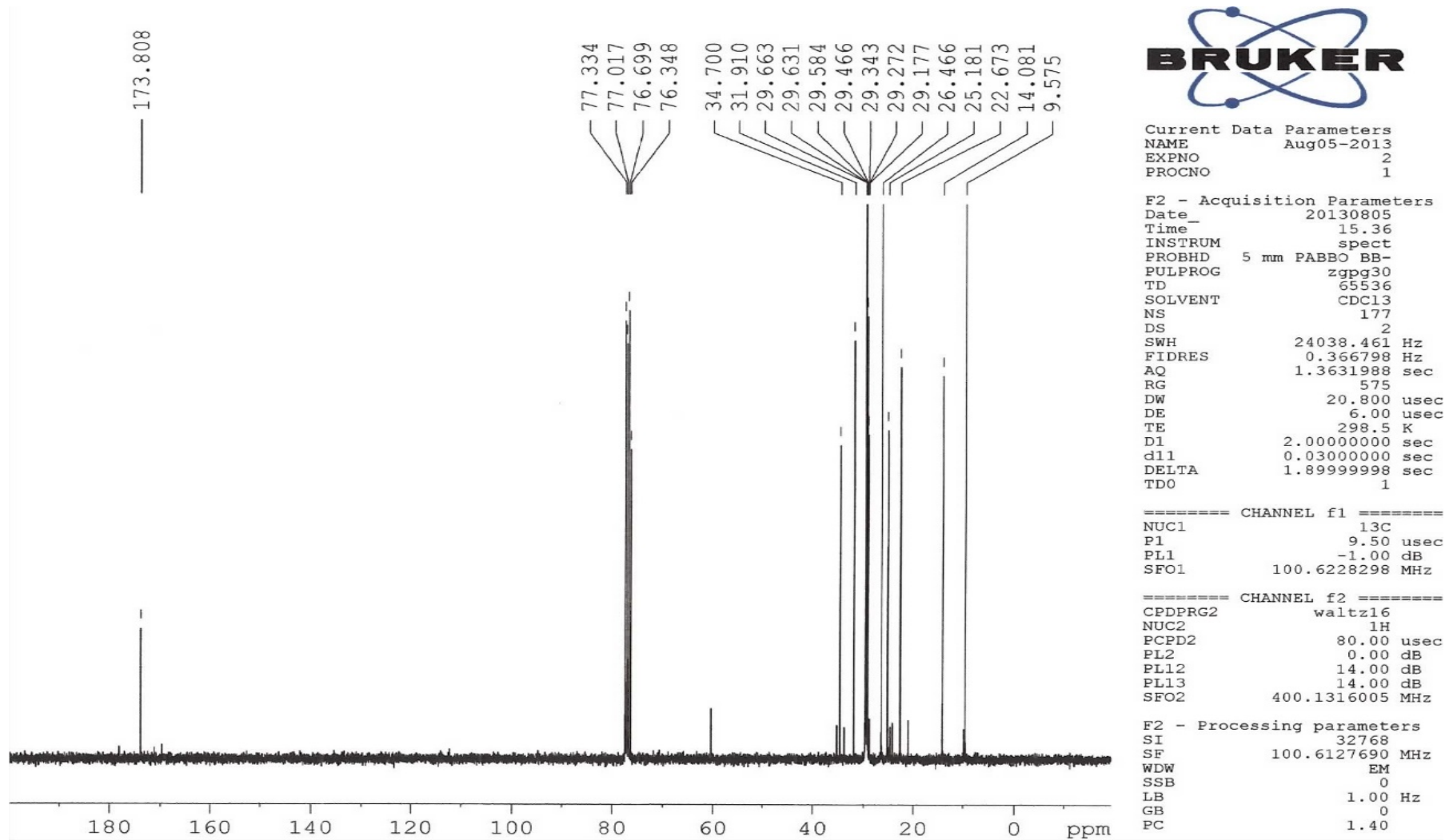
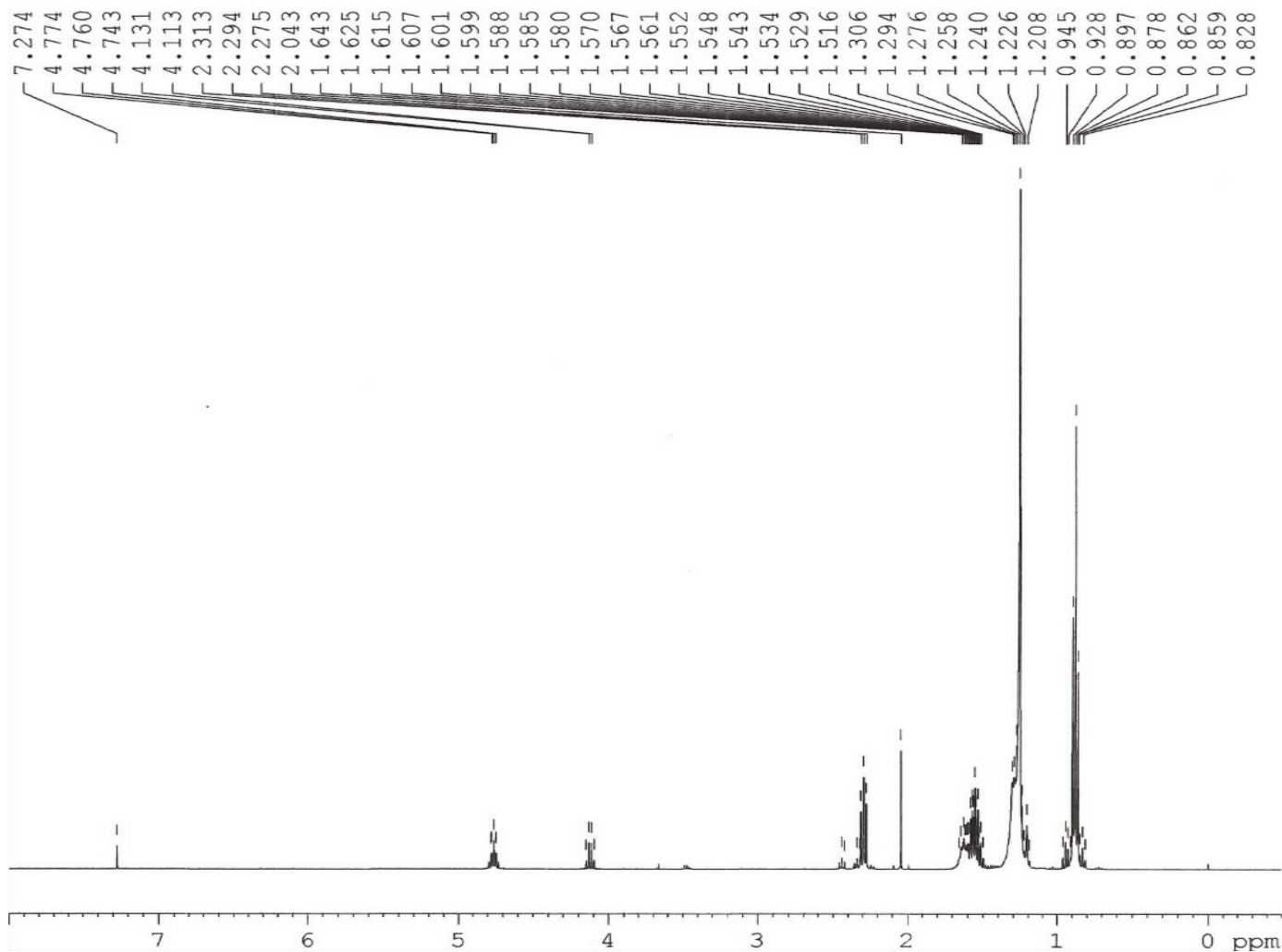


Fig. S4. The NMR spectrum of 3-pentyl laurate CMR



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 PROCNO 1

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F2 - Processing parameters
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Fig. S5. The NMR spectrum of 3-pentyl myristate PMR

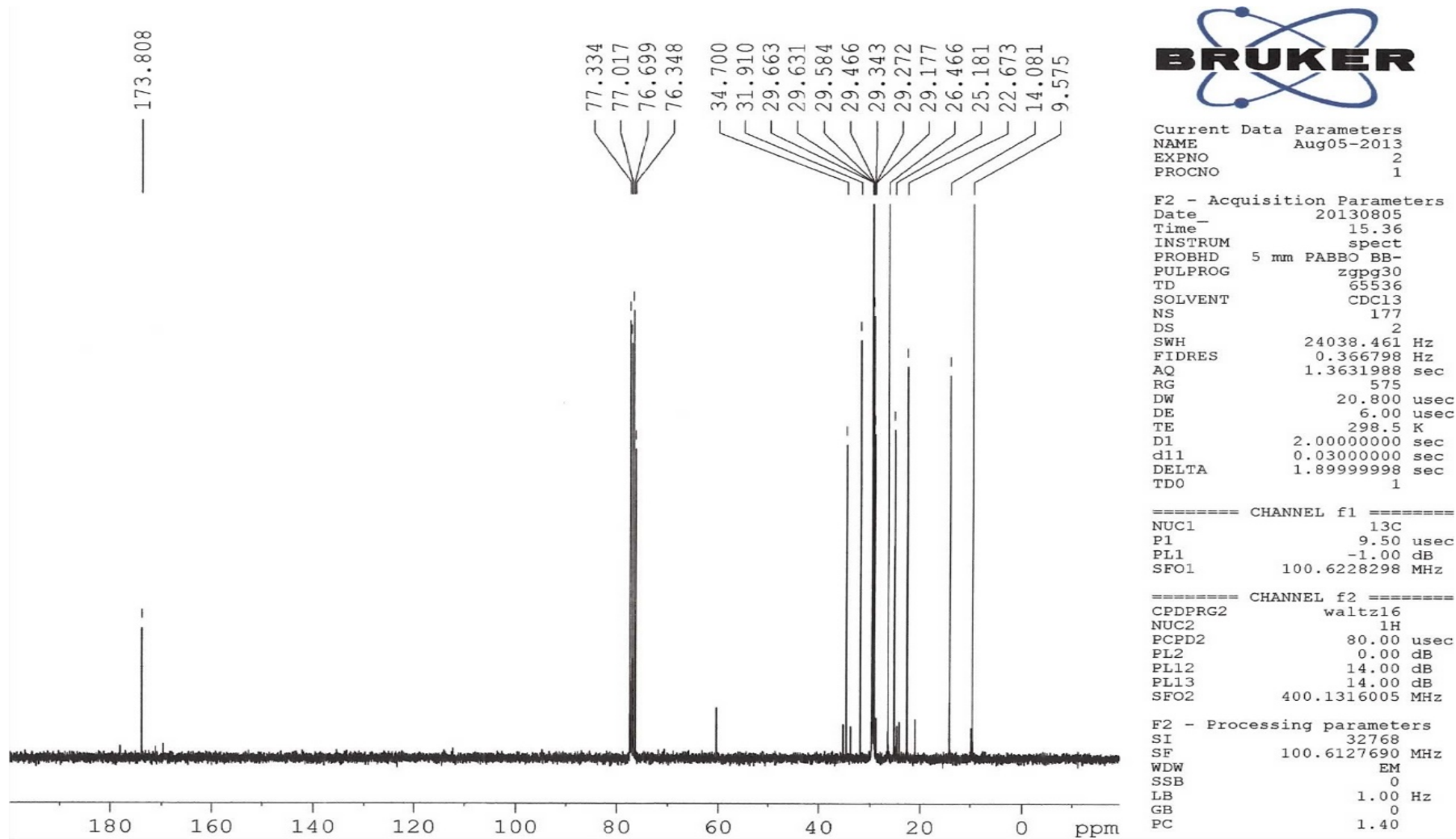


Fig. S6. The NMR spectrum of 3-pentyl myristate CMR