# Chemoecological Studies on the Reproductive Behaviors of the Darkwinged Fungus Gnat, *Bradysia paupera* (Diptera: Sciaridae)

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# Chapter Ⅰ Introduction

In recent years, an increasing consumer demand for commercial mushroom products recognized as natural healthy foods has raised concerns about problem in mushroom production. Sciarid flies trouble mushroom growers as a serious pest of mushrooms by daily damaging crops. This problem is increased with a lack of optimal pesticides for conventional use because of increase in the strictness of pesticide regulations amid concern of environmental protection (Finley et al., 1984; Ishitani, et al., 1997; Gotoh et al., 1999; Jess and Kilpatrick, 2000).

The number of Sciaridae species in the world is unclear, but in North America alone, 150 species have been reported (Harris et al., 1996). Most species are causing worldwide massive economical loss to both mushroom and horticultural production, mainly in the genera *Lycoriella* and *Bradysia*. In Northern Ireland annual losses have been estimated at $1.2 million (Jess and Kilpatrick, 2000). In Chiba Japan, annual production of 900,000 kg mushroom are frequently affected by the occurrence of sciarid flies (Sasagawa, 1993; Ishitani et al., 1993; Gotoh et al., 1999).

To control sciarid flies, various efforts including fumigant (Cantelo, 1989), IGRs (King, 1991), biological agents *Bacillus thuringensis* (BT) (Osborne et al., 1985), the predecious mite *Hypoaspis miles* (Wright and Chanbers, 1994; Ydergaard et al., 1997), the parasitic nematode *Steinernema feltiae* (Gouge and Hague, 1994) and the parasitoid wasp *Synacr* sp (Hellqvist, 1994) have been used. However the effectiveness of these measures is not sufficient (Jess and Kilpatrick, 2000). Additionally, easier accumulation of pesticide residues in mushroom fruit (Sato and Asawa, 1995; Sato et al., 1995) and development of pesticide resistance subtype (Brewer, 1990; Bartlett and Keil, 1997) have been reported. In Japan, sticky fluorescent black lights emitting visible blue and UV light (Ishitani et al.,1997) are normally used for monitoring and trapping adult sciarid flies, but black lights may stimulate fly activity and inhibit fungus development (Ishitani, 1997; Jess and Kilpatrick, 2000).

Therefore, it is a situational necessity for growers working in mushroom industry to explore new options to control these troubsome flies. The new maneuvers are expected to come for pesticide residue free procedures. Pheromones are now being used to disrupt mating in a wide range of lepidopteran pests in agriculture, horticulture and forestry, and offer farmers, growers and foresters season-long, user-friendly and environment-friendly protection for their crops

(Jones, 1998). Therefore, elucidation the potentials of the key factors mediating intra-specific communications of the reproductive behaviors of the sciarid flies, development of new practicable strategies are expected to eventually build up an integrated pest management system.

However, identification of semiochemicals including sex pheromones from small bugs such as sciarid flies requires arduous work. Therefore, relatively little is known about the chemical factors regulating sexual behaviors in sciarid flies (Diptera:Sciaridae). Until now the existence of sex pheromones involved in mating behavior have been reported in only 3 species (Kostelc et al., 1979, 1980; Alberts et al., 1981; Gotoh et al., 1999). In 1979, Kostelc et al. reported the main sex pheromone of *Lycoriella mali*, but later the sex pheromonal effects of n-Heptadecane was questioned by Gotoh et al (1999).

*L. mali* Fitch appears to be the most common species seriously infecting mushroom (Finley et al., 1984; Sasagawa, 1993). Since this species was studied by other research groups, this dissertation centers on *B. paupera*.

For many years sciarid flies including species in the genus *Bradysia* were considered merely a nuisance, but fungus gnats appear to successfully develop on fungal sources as temperature moderated greenhouses have been widespread. *Bradysia* species commonly inhabit moist shady areas within woodlands, greenhouses, mushroom pollard, and field crops, living in the area between the soil surface and lower foliage (Harris et al., 1996; Springer and Carlton, 1993). They are frequently the cause of significant losses in the production of plant cuttings and seedlings, and commercial mushrooms. Ornamentals and vegetables can also be substantially damaged. Larvae of fungus gnats can directly feed on commercial mushrooms and plant roots. Moreover, the experimental confirmation of fungus gnats as potential disseminators of plant pathogens (Kalb and Millar, 1986; Jarvis et al., 1993) has elevated their status to serious pest.

The adults of *B. paupera* are small (average length of males is 2.5 mm, females 3 mm) and inconspicuous (Fig. 1-1 life cycle of *B. paupera*). Steffan (1966) reported the following morphological characters to distinguish the genus *Bradysia* from other genera: "maxillary palpi three-segmented, usually with sensory pit, protibiae with preapical ventral unilateral comb separated from general tibial vestiture by triangular bare area, metatibiae with two subequal spurs, and posterior wing veins bare."

The current research was conducted to elucidate semiochemicals that regulate reproductive communications of *B. paupera* by elucidation of the relationships between chemical cues and reproductive behaviors, further identification of

bioactive components and examination the effects of the identified chemicals on the behaviors of the flies.

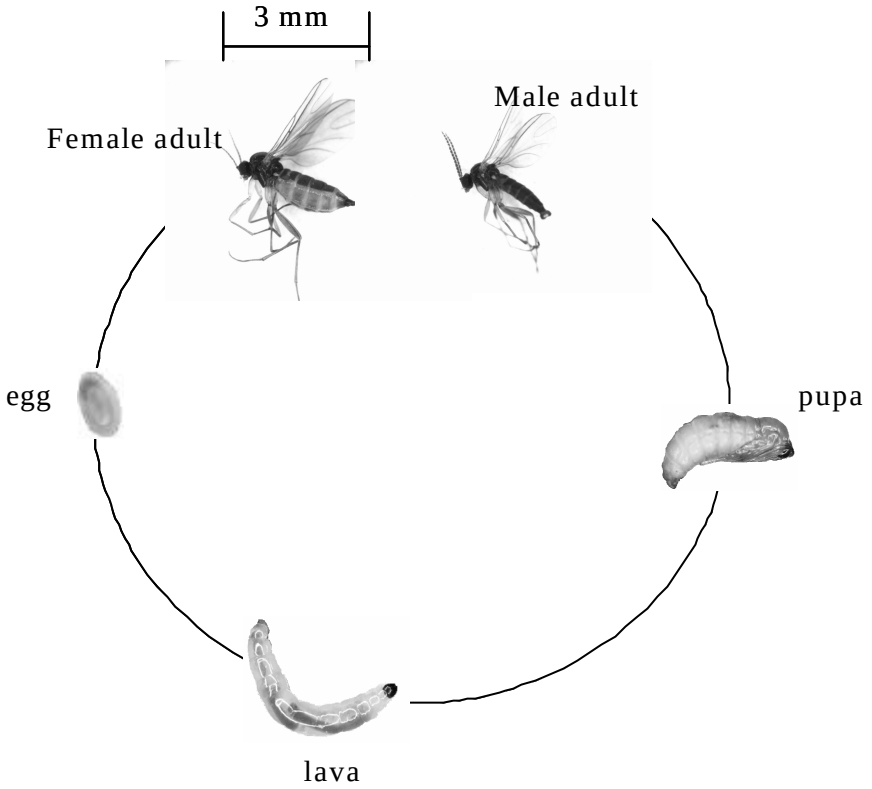



Fig. 1-1. Life cycle of fungus gnat, *Bradysia paupera* TUOMIKOSKI (Diptera: Sciaridae)

This work is reported in the following 5 parts: 1) establishment of a laboratory colony (Chapter Ⅱ) to ensure the supply of insects, 2) observation of sequential mating behaviors and use of bioassay methods to confirm chemical cues involved in mating behaviors (Chapter Ⅲ), 3) modification of GC-EAD (Chapter Ⅳ) for small sized antenna to help monitor the active components, 4) identification of the active sex pheromonal components (Chapter Ⅴ), and 5) the significance of the study is discussed (Chapter Ⅵ).

## Chapter Ⅱ. Establishment of Laboratory Colony

The sciarid flies were collected from a mushroom production house at Unakami-Machi in Chiba Prefecture (Japan), and colonies maintained in our laboratory. In order to culture the flies effectively and to provide a stable qualitative insect source, life history and biology of this fly were investigated prior to the

establishment of the rearing method.

Some *Bradysia* species e.g. *B. corophil, B. impatience, B. tritici* have been studied as well as the yellow fruit fly, *Drosophila melanogaster*, because of genetic characteristics to produce monogenetic progeny (Metz, 1938). Therefore, laboratory rearing methods of these species have been established (Harris et al., 1996). Hudson et al. (1974) employed two methods for laboratory rearing of *B. paupera* colonies to evaluate the efficiency of a pathogenic nematode on sciarid fly populations. They mass reared colonies by maintaining flies in muslin cages with manure and dried animal blood, and fed small scale colonies by maintaining flies in glass tubes with agar and grain seedlings. These rearing methods seemed to be appropriate for culturing *B. paupera.*

However, the focus of the current study was the chemical ecology of this fly and requires avoidance of every possible type of contamination in the sampling process. Any substances other than from the insect itself such as the manure diet and dried animal blood should not be transferred to the rearing chamber. In addition, the food material may affect the properties of the pheromone (Ponomarev et al., 1997), thus the above mentioned rearing methods were thought improper for our purpose. Gotoh et al. (1999) used a method to rear the *L. mali* mushroom fly to study sex pheromones. This method was considered proper for culturing *B. pauper*, because the mushroom flies were fed fungus diet similar to the natural food source of the flies.

On the other hand, the biology of *B. paupera* remained somewhat unclear in previous studies, e.g. whether the female fly of this species produces unisexual offspring, the longevity of the adults, sex maturity, sex ratio and affection of temperature on development. This knowledge is important for this study and may be acquired by a moderate rearing method.

The establishment of the laboratory colony was accomplished by rearing single flies in glass dishes for biological observation and culturing a mass population in plastic boxes to maintain a steady supply.

## Materials and Methods

Single rearing

Eighty glass dishes (Dia. 50 mm, Hi. 15 mm) were washed and baked at 200℃ before use. Dishes were divided into 4 groups of 20 each of A, B, C and D. a moist filter paper was matted to the bottom of each glass dish, then a wetted cotton stick and a piece of mushroom fruit (about 1 $cm^3$, Shiitake *Lentinus edodes* ) were placed on the filter paper. No mushroom material was supplied to group D for observation

of survival with no food. Pregnant female flies obtained from the mass culture colony (see mass culture) were singly transferred into prepared glass dishes for egg deposition. The dishes with female flies were incubated at 17 ± 2℃(group A), 20 ± 2℃(group B) and 25 ± 2℃ (group C and D) under 2:22 L:D conditions. Each day the developing stage and status of the flies were recorded until adult eclosion.

Separately, propagating mycelium (1 cm×1 cm×2 mm) obtained from block medium (Shiitake, *Lentinus edodes* ) were put on the center of the moist filter paper in each of 20 dishes to determine whether the female flies prefer to oviposit on fungus. Only wetted filter paper was placed in dishes as a control. Twenty six pregnant females were each singly transferred into these dishes, then placed under 2:22 L:D conditions at room temperature (25 ± 2℃). After 3 days, the oviposition location and the number of the eggs in the dishes were recorded under a stereoscopic microscope by placing the dish on a paper grid (5 mm×5 mm/mesh). The distribution of the eggs was recorded by location in 64 small squares .

Massive culturing

An artificial block medium formed by mixing sporophores (Shiitake, *Lentinus edodes*) in sawdust and rice bran (11 cm×18 cm×13 cm, Aoyama Mushroom Center) was placed in a kitchen plastic box (33 cm×44 cm×23 cm, Sekisui House). To increase space and adjust moisture, adequate woodchip beddings (about 2 cm thick) (White break, Charles River LTD. Co. Japan) were also add to the box. The artificial block medium and woodchip beddings supplied moisture and provided propagating mycelium, mushroom fruit or wood fibers as foods for growing larvae and a place for oviposition. A paper towel (Kim Towel) was inserted into the container lid to prevent lavae or adults from escaping. In the center of the lid, a window (13×16 cm) was opened, covered with gauze and lap film so that the humidity in the container could be adjusted by opening or shutting the window. About 50 pairs of flies were transferred to the plastic box containing block medium and incubated at 17 ± 2℃ and 25 ± 2℃ under a 2L:22D regime until the mushroom fruit developed about 10 days later. Some matured mushroom fruit was removed and preserved at 5℃, as storage food to supplement the larval diet when lack of fungus food in the box. Periodical disposal and cleaning of rearing box were conducted after it last about 4 months.

## Results

Single rearing

In a total of 80 dishes, 65 dishes contained flies that finished development from egg to adult, while flies in 15 dishes ended with death, including 13 dishes of

group D. The adults in every dish were unisexual, thus confirming *B. paupera* is also monogenic.

A large variation occurred in the developmental period under the different temperatures (Fig. 2-1). Dishes of groups A-C showed average periods of development from egg to adult of 29 days at 17℃ (group A), 24 days at 20℃ (group B) and 18 days at 25℃ (group C), but variance in adult emergence between precocious and serotinous flies was 11 days at 17℃, 6.5 days at 20℃ and 5 days at 25℃. Therefore,an overlap of developmental stages of larva pupa and adult, were seen after culturing for 28 days at 17℃. However, the variance was comparatively lower for 5 – 6 days at 20℃ and 25℃.

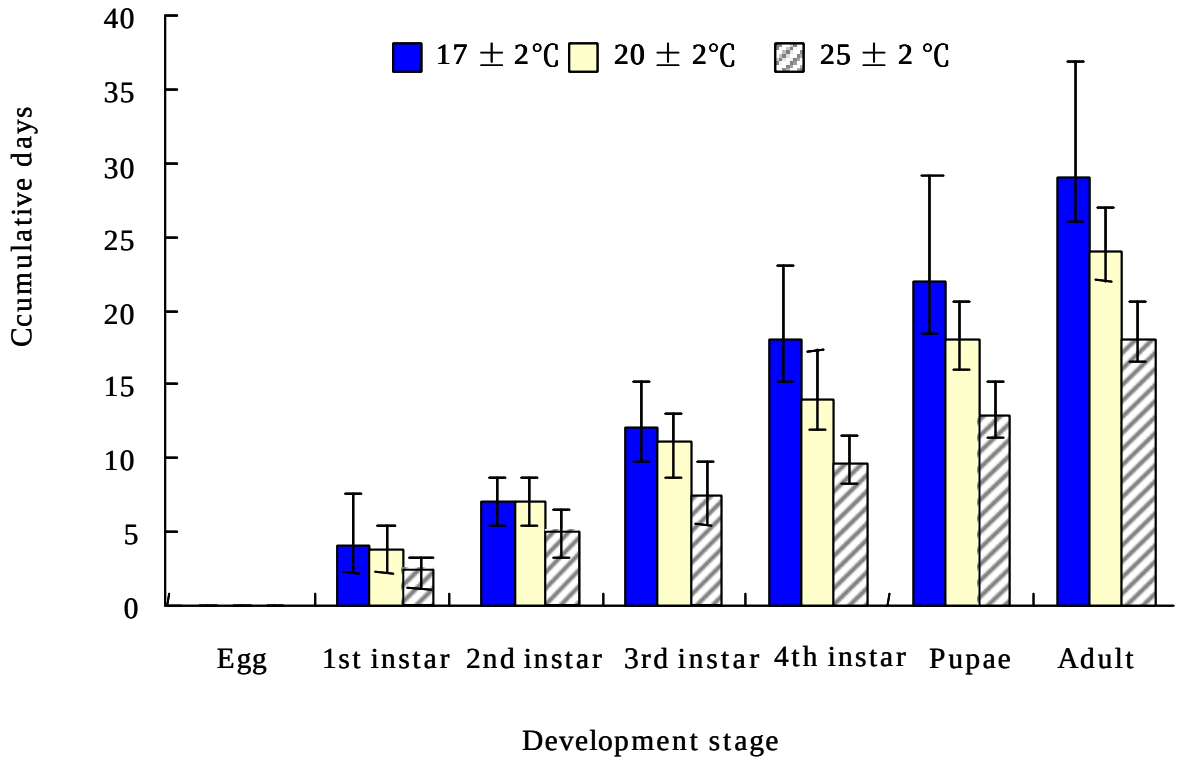



Fig. 2-1. Development of *B. paupera* under different temperature conditions.

In group D with no food, surviving larvae fed on dead mother's body or debris of the filter paper to reach the adult stage at 2 days more early late. But the body size was obviously smaller than flies fed on mushroom.

In all observations, males emerged 1 d prior to the females. Sex ratio of female to male offspring was 38 dishes to 27 dishes. Female offspring occurred about 1.4 times more than male offspring.

The longevity of the adult was from 2 – 7 days under humid conditions, but only 1 day under dry conditions. One hundred females and one hundred males were

randomly selected and weighed. Females were averaged 0.31 mg and males 0.18 mg.

Preference of fungus materials

Of 20 treated dishes 18 contained eggs (Fig. 2-2). In dishes with mycelium diet, 84% (1370) of the total 1633 eggs were on the mycelium at the center of the dishes, and averaged 90.7 per dish. In dishes with only filter paper, a total 296 eggs were deposited randomly around the edge of the filter paper or in apertures, and averaged 49 eggs per dish. Egg numbers in the controls were obviously low. Thus the female flies showed a clear preference to lay eggs on mycelium, but when no fungus was available the ovipositors prefered to conceal the eggs in the wetter creases.

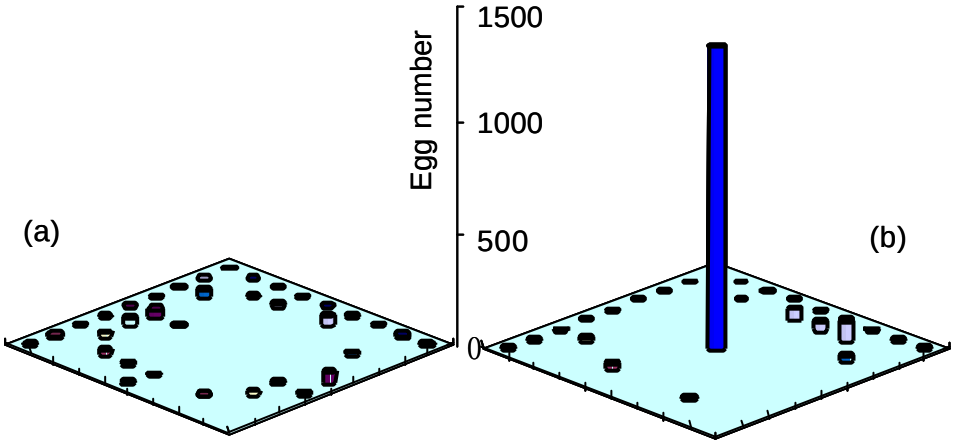



Fig. 2-2. Distribution of eggs laid by females in dishes with filter paper alone (a), and with mycelium (*Lentinus edodes*) in center of the dishes (b).

Massive culturing

One rearing plastic box with one block medium yielded ca. 5, 000 flies per month, and 3 to 4 generations could be reared. The plastic box rearing method provided year rounds rearing and daily supply of the insect.

## Discussion

Careful dish rearing clarified that *B. paupera* is a monogenic producing unisexual progeny. The mechanism of monogenic was shown by Metz (1938) and other researchers (Harris et al., 1996) to occur with elimination of one or two X chromosomes at the seventh or eighth cleavage of the zygote. If one chromosome is eliminated, the somatic cells will be X'X and the individual will develop into a

female. If two chromosome are eliminated, the soma will be XO and result in development of a male. Most species in *Bradysia* e.g. *B. inpatiens* and *B. coprophila*, produce unisexual offspring while some species e.g. *B. reynoldsi* and *B. tritici* do not. The significance of the production of unisexual progeny may be a means of insuring out crossing (Harris et al., 1996). This same meaning is considerable for *B. paupera*. This monogenic characteristic can be used to easily obtain any sex pupa or adult in individuals as needed.

The life span of one generation of *B. paupera* was clearly distinct at different temperatures. The developmental times from egg to adult of *B. paupera* were 18 days at 25℃ and 29 days at 17℃. This developmental rate is very similar to the developmental period of *B. impatiens* (6.3 days at 20℃), *B. coprophil,* (19.9 days at 23.9℃) and *B. tritici* (22.2 days at 20℃) (Harris et al.,1996). Terefore, the developmental time of the flies observed in the dishes possibly reflects the normal growth rate.

A considerable variation of developmental times was observed between individuals even in the same dish at the same temperature. Therefore, temperature, food competition and genetic differences (although from the same mother) also may be factors affecting developmental time. In a review on Bradysia, Hellqvist et al. (1996) also suggested that highly variable developmental times when reared on the same diet is a characteristic of Bradysia.

In mass rearing during this study, woodchip beddings added into plastic box as to absorb excessive moisture resulted in the reduction of dew condensation. The addition of wood powder was found to be quite effective for maintaining the appropriate balance of water in the diets of the mass rearing for yellow peach moth *Dichocrocis punctiferalis*. by Honda et al. (1979), Similar result showed also the codling moth, *Carpocapsa pomonella* benefited by using sawdust for mass rearing (Brinton et al., 1969). The addition of woodchip beddings contributed to largely reduce number of deaths in adult *B. paupera* flies were trapped by drops of dew on the inside wall of the box. In addition to adjusting the humidity inside the box, woodchip beddings also provided more space for larvae to dwell.

*B. paupera* exhibited good development in a mass rearing box at a high temperature such as 25℃. However, rearing at such a high temperature was not appropriate because it also allowed outbreak of various germs such as penicillium and quick deterioration of mushroom block medium, Although rearing at 17℃ extended the development time and resulted in a large overlap of different stage forms, appropriate for conveniently collecting all stages of the test insect any time, 17℃ was appropriate for room culturing.

Male flies of this species emerged 1 d prior to females. The same observation was reported for other *Bradysia* spp. (Steffan 1966, Kennedy1974). Males usually live longer than females, which die soon after oviposition. Because adults are short-lived (approximate1y 3 d), earlier emergence of males may increase the chances of copulation (Harris et al., 1996).

Larvae of this species survived even in dishes with no food by eating dead mothers. This phenomena also occurs in other *Bradysia* species. Gardiner et al. (1990) and Harris et al. (1996) reported larvae primarily feed on fungi and can be cannibalistic. Kennedy (1974) observed *B. impatiens* larval survival was reduced when fungal abundance was low. However, none of my observations showed larvae feeding on each other, despite very poor survivorship. Female adults appear to prefer to deposit eggs near the mycelium and feed on the mushroom diet. Therefore, it is essential to add mushroom diet to culture flies.

Single rearing and box massive culture showed reliability for stable supply of quality insects.

## Chapter Ⅲ. Key Factors in Controlling Mating Behavior

Female *B. paupera* produce unisexual offspring, the offspring grow up to become adult again they need to find their mates laid by different female thus may be at distance away from each other to copulate and produce next generation. Then how does a fly find or recognize a mate? What are the behavior to achieve courtship when he or she recognizes the existence of a mate? Further, what triggers sexual behavioral responses in this fly? Answers to these questions will make it possible to evaluate the potential of semiochemicals and feasibility of using those chemicals to establish a management program for *B. paupera.*

In order to accomplish such a program, the proper bioassay methods are required for evidence of chemical cues functioning in communication, and to determine the relationship between bioactive components and characteristic behavioral responses. The proper bioassay method should be able to repeatedly observe the evidence of a relationship under stabilized conditions (Baker and Cardé, 1984; Howse, 1998). Inspection of the possible influence of physiological conditions and environmental conditions on behavioral responses is also necessary.

In this chapter, for evidential analysis of the relationship between chemical cues and reproductive behavior, the mating behavior of the fly (Section 1) was observed, followed by investigations on biological properties of the sex pheromone

(Section 2), and effect of environmental factors and light intensity on the male responses to the sex pheromone (Section 3).

## Section 1. Mating behavior and existence of sex pheromone

### Materials and Methods

Observation of mating behavior

A glass tube (125 mm × 30 mm) covered on both ends with polyethylene muslin, inside humidity maintained with a moist cotton wool, was used to observe mating behavior. One to 2 day old virgin females obtained by the single rearing method were paired with 1-3 day old unmated males in a glass tube. All courtship activity was recorded. The observations of mating behavior were conducted at room temperature (22-25℃) under fluorescent light (12 W ca. 500-1000 lx).

To describe mating behavior similar words e.g. wing fanning (WF), walking in an abrupt zigzag approach (ZA), curling abdomen (CA), clasping and pivoting 180 degrees (P 180) and mating occurring (MO) as indicators, have been used by Alberts et al. (1981) for *B. impatiens*, Gotoh et al. (1999) for *L. mali* and Sasagawa (1985) for *B. agrestis*. In order to be convenient and consistent, these terms and abbreviations were used as descriptors for the behavior of *B. paupera*.

The detail behaviors, categorized as WF, ZA, CA, P180 and MO as the mating behavioral components, were observed for a total of 75 pairs of flies.

Each observation was carried out within 10 minutes and another 5 minutes post-copulation behavior was continually observed after the first copulation ended.

Existence of female sex pheromone

A moist cotton pad was matted on the bottom of a plastic box (Dia. 15 cm×Hi. 9 cm) with a window (3 ×4 cm) on the roof covered with a polyethylene muslin (T-900, TOREY) (Fig. 3-2-1-a, group assay). Twenty to thirty 1-2 day old males were transferred into the plastic box. After 2 min adaptation, the males were exposed to females confined in a porous plastic medical capsule (7 mm×10 mm) or female extract loaded on a small filter paper. The responses of males was observed and recorded for the five behavioral components WF, ZA, CA, stimuli source oriented flight (OF) and landing on stimulus source (LO) for 3 min. This bioassay method with a group of males in a cage will be referred to as a group assay throughout this report. All group assays were conducted at 25 ± 2℃ under a fluorescent lamp (ca. 500-1000 lx).

Female crude extracts were prepared by dipping 100 female flies anesthetized

at -10℃ for 5 min into 3 ml hexane for 2 h. Solid residues were further subjected to Soxhlet extraction for 2 h to extract as completely as possible the hexane soluble substances within the insects. The female crude extracts were concentrated to 4 female equivalent /μl (FE) under a $N_2$ stream, and held at –20℃ for later use.

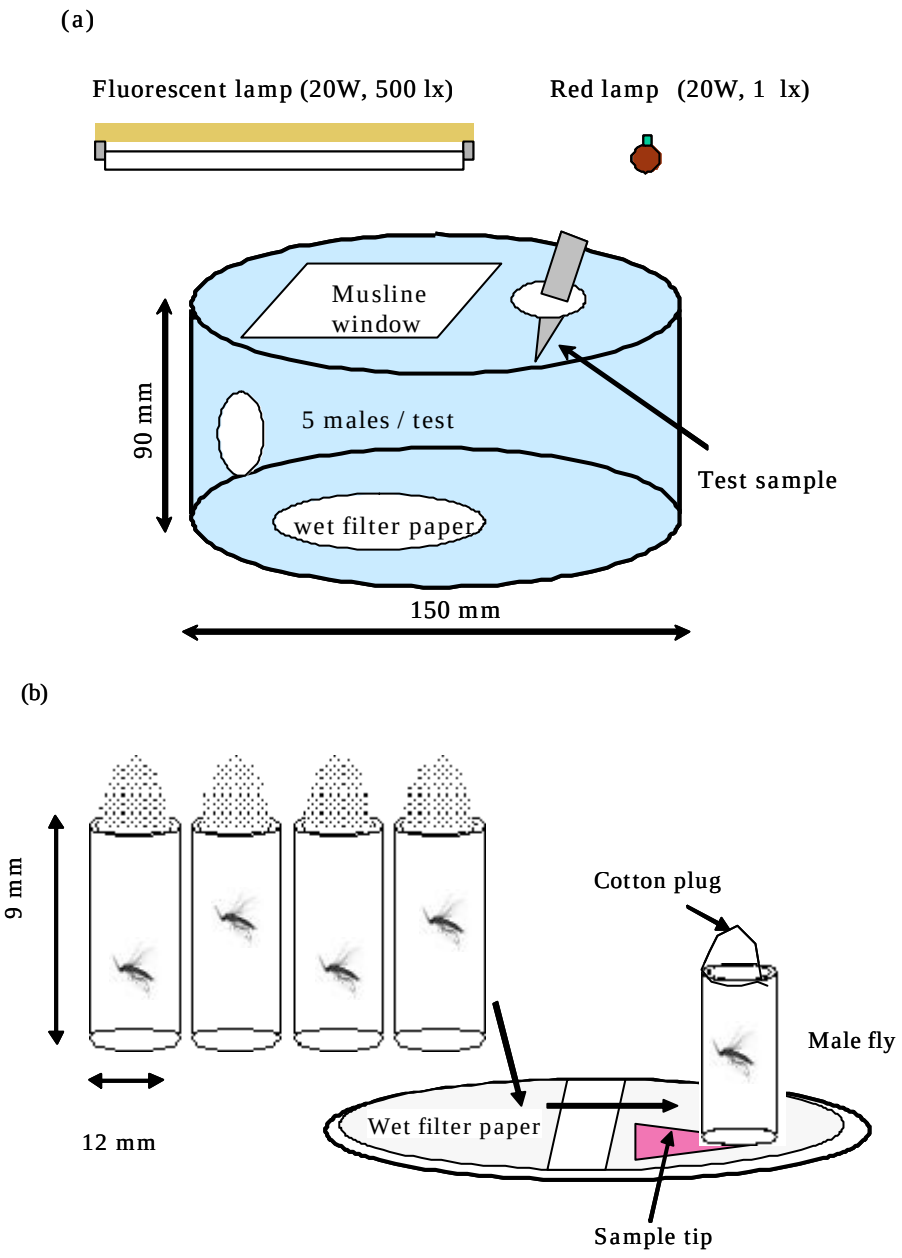



Fig. 3-2-1. Schematic of bioassay methods. See text for detailed explanation of group assay (a), and individual assay (b).

Four 2 day old females were immobilized at -10℃ for 3 min then placed in a

porous basket. The basket was hung in the cage for observation of the responses of 28 males. Four Soxhlet extracted female bodies were treated with 4 FE of female extract and put into a porous basket. Males (32) were exposed to the basket in the cage for observation. As a control, 4 Soxhlet extracted female bodies were similar exposed to 28 males and responses observed in males.

## Results

Mating behavioral sequence

In the observed 75 pairs (Fig. 3-1-1), 43 pairs copulated. The mating behavior

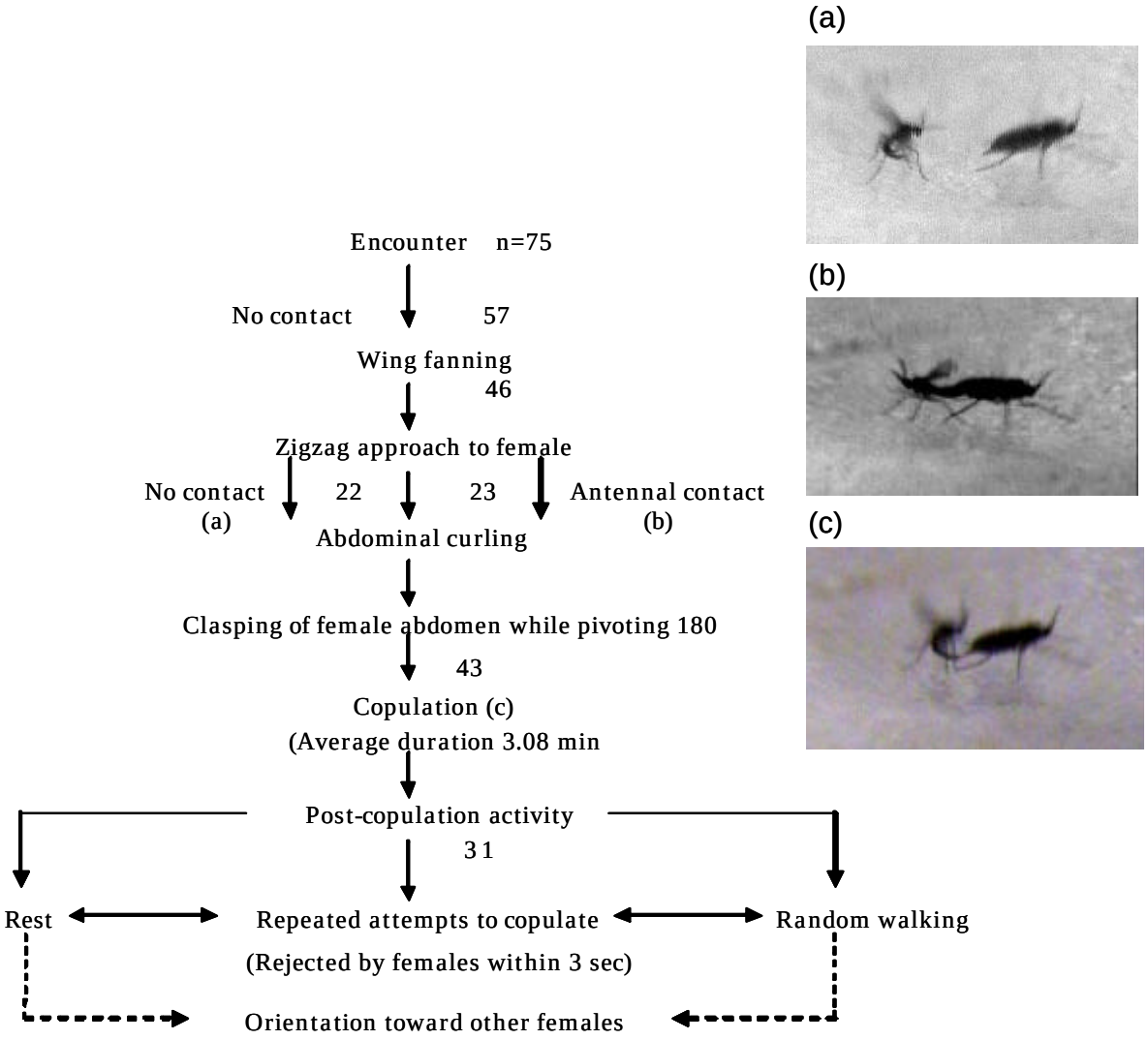



Fig. 3-1-1. Behavioral sequence in the mating of *B. paupera*. Arrows show steps of the sequence. Arabic numerals give the number of males that elicited each descriptive behavior. Dashed lines with arrows indicate the possibility of males to mate with other females. Three photographs show behaviors corresponding to parenthesized a, b, and c.

was composed of the following behavioral steps. After recognition of the female, the male at first started intermittent wing fanning (WF), then walked near the female by a zigzag approach (ZA) while continuing WF. After becoming very close to the female, the male curled the tip of his abdomen (CA) forward beneath the thorax (Fig. 3-1-1-a). Of the males showing these behavioral components, 23 males clearly showed CA after contacted the females with antennae or legs, but another 22 males showed abdomen curling without contacting the females (Fig. 3-1-1-b). Next the male grasped the abdominal tip of the female with his claspers while pivoting 180 ° (P 180) around body axis to form a tip to tip linear shape and mating occurred (MO) (Fig. 3-1-1-c). In all observations only the male showed the above typical behavioral pattern. No typical calling or pursuing of the male behavior was observed in the female. Copulation lasted on a average for 3 minutes, the shortest was 2 min and 18 sec and the longest was 6 min and 19 sec.

After copulation, the males rested briefly or walked around and 31 males attempted to copulate again. The mated females usually refused to re-copulate by running away or kicking the male off with her hind legs.

Existence of female sex pheromone

Responses of males to virgin females confined in a basket are shown in Fig. 3-1-2. Among 28 males, WF (57.1%), ZA (28.6%), orientation flight (OF)(67.9%) and landing on the origin of the stimulant (64.3%) were observed. The responses of males to physically isolated females were almost the same as that observed in the mating behavioral sequence. The responses to hexane washed female bodies was obviously low (WF, 0%; ZA, 0%; OF, 13% and LO, 5.6%), but when extract of females was applied to the washed female body, activity was revived to 31.3% for WF, 34.4% for ZA, 50% for OF and 34.4% for LO.

## Discussion

In observation of mating behavior, male *B. paupera* showed a sequence of behaviors including WF, ZA, CA, P180 and MO. The sequence of these behaviors was performed almost the same by all males which successfully copulated. Alberts et al. (1981) described this sequence of mating behaviors as stereotyped in their observations on *B. impatiense*. Similar mating behaviors were observed in *Bradysia agrestis* by Sasagawa (1985). Thus beginning with wings fanning while approaching to a female in a zigzag pattern, the male fly catching the abdomen of the female and pivoting 180 degree, until mating occurs are perhaps the typical mating behaviors of all Bradysia species.

After *B. paupera* males perceived presence of a conspecific female, they

started wing fanning and zigzaged walking even without contacting females. This suggests contact is not necessary to evoke WF and ZA behavior in males. Some other factors e.g. volatile chemicals, visual or acoustic cues may stimulate these behaviors.

When a female was confined in a porous basket, it was impossible for the males to perceive visual stimulus from the females, but they could still orient to the females. Therefore visual stimulation appears to not be important for male behavioral responses.

Wing fanning of males may serve as an approach song and be related to acoustic communication in mushroom fly mating (Kanmiya, 1999). However, female *B. paupera* showed no typical calling behaviors while males showed the typical mating behaviors. WF of males may make it easier of less active females to accept the copulative attempts of males, but WF is likely not responsible for male orientation to females.

Restoration of sex pheromonal activity to elicit WF, ZA, OF and LO by reapplying hexane extracts of females to washed females is vital and important evidence that female bodies which had lost pheromonal activity after Soxhlet washing. This fact demonstrats that no visual or acoustic stimulus from females is needed to evoke pheromonal responses in males and suggests of airborne factors, such as volatile female pheromones trigger male sexual behaviors.

## Section 2. Functions and properties of the sex pheromone

### Materials and Methods

Responses to test samples was observed by two methods: (1) using one test male (individual bioassay) and one test with over 20 males (group bioassay).

Group bioassay in cage

The method of group bioassay was as previously described in section 1 of this chapter, and shown in schematic Fig. 3-2-1a.

Individual bioassay in tube

In individual bioassays, male flies were singly kept in a plastic tube (ca. Dia. 10 mm×Hi. 45 mm) of which the top end was plugged with cotton and other end was capped with polyethylene muslin (Fig. 3-2-1-b, individual assay). In each assay over 20 males were used. Before bioassay, the tube with the male was placed on a wet filter paper for adaptation and drinking in 5 min. The tube was moved to another dry filter paper and the opening side covered on a test sample (e.g. a small triangular filter paper treated with extract, or fly bodies). Then the male responses

were observed for 2 min. Behavioral responses of the male were recorded as WF, ZA, and CA. Newly recruited males were used after a day long bioassay. All the bioassays were conducted under a fluorescent lamp (ca. 500-1000 lx) at 25 ± 2℃).

Sexual maturity of females and males

Individual assay was employed to analyze the dynamics of the sex pheromonal activity as related to the sexual maturity of female and male flies. The developmental stages of females from pupa to matured adults were divided into 7 stages: 1) immature pupae of white color, 2) matured pupae of brown color, 3) 0-5 min after emergence with white abdomen, 4) 30 min after emergence, 5) 2 hours after emergence, 6) 1 day after emergence and 7) 3 days after emergence. To prevent the female from moving, double sided sticky tape was used to slightly fix the fly to the filter paper. Twenty 1 day old males were used in the observations.

Sexual maturity of male flies was investigated by observing the responses of the males to females in 5 different male stages: 1) just after emergence (10 males), 2) 30 minutes after emergence (14 males), 3) 2 hours after emergence (10 males), 4) 1 day after emergence (10 males) and 5) 3 days after emergence (20 males).

Pheromonal activity of crude extract

In order to examine the response of males to different amounts of female extracts, the individual assay was used. Response of males to female crude extracts of $2\times10^{-3}$ FE, $2\times10^{-2}$ FE, $2\times10^{-1}$ FE and 2 FE were separately checked by recording individual WF, ZA and CA. Male extracts were also prepared by the same method.

Persistence of pheromonal activity

The individual assay was used to access the persistence of pheromonal activity. Twenty 1 day old females were divided into 4 groups and refrigerated at -10℃ for different lengths of time. The frozen female flies were moved from the refrigerator after 1) 5 minutes, 2) 2 hours, 3) 3 days and 4) 30 days to check the pheromonal activities by exposing them to twenty 1 day old males separately. Another 10 I day old females were kept at -10℃ for 1 h then removed and exposed to the air, two halves of 10 dried female bodies were tested for activity after 3 days and 6 days.

The location of the female sex pheromone

The individual assay was used to monitor the distribution of the sex pheromone on each part of the female body. Four 1 day old female flies were kept at -10℃ for 5 min for anesthetizing, then the immobilized bodies were dissected into head +antennae, thorax + legs, wings, and abdomen under a stereomicroscope. The 4 body parts were examined for sex pheromonal activity separately with 10 males.

Further, twenty 1 day old females were dissected into the same 4 body parts

and separately extracted with 1 ml hexane for 30 min. Bioactivity of 4 FE of the extracts of each body part were tested.

The factors triggering male abdominal curling

The Individual assay was employed to analyze factors triggering male abdominal curling behavior. Wings of 100 each one day old males and females were removed under a microscope after flies were anesthetized by refrigerating at -10℃ for 5 min. Dissected wings were extracted in 1 ml hexane for 2 h, then the extract was adjusted to 5 wing equivalents (WE) /μl. A total of 5 bioassay samples were made as testing samples: 1) 8 female wing equivalents (FWE), 2) 8 female wings, 3) 10 male wings, 4) 10 male wings coated with 10 FWE, 5) 10 male wings placed at the side of a triangular filter paper coated with 10 FWE and response checked by 1 day old males.

Wind tunnel test

An acrylic cylindrical wind tunnel (L. 200 cm ×Dia. 30 cm), consisting of 4 sections of each 50 cm, was used to test distance attractiveness of the female sex pheromone (Fig. 3-2-7). The wind tunnel model was a device able to condition air and vent air from wind tunnel. Lures were 5 FE of female crude extract applied on a small triangular filter paper and fixed to a small sticky trap (10 cm ×5 cm×4 cm) made of plastic film. The small sticky trap was placed on a stand upwind of the wind tunnel about 10 cm from the upwind end. In each test, twenty 1-2 day old males randomly captured from the mass culturing colonies were released from a release point 180 cm away from the lure. After 3 min and 8 min of release the location of each free male was recorded in terms of position in the section or whether trapped at that time. All tests were conducted at a flow velocity of 25 cm/sec at 25 ± 2℃ under a fluorescent lamp (500 lx). Each treatment was tested 3 times. As a control hexane treated filter paper was substituted. The significantly difference from control was analyzed by Wilcoxon t-test at $P<0.05$.

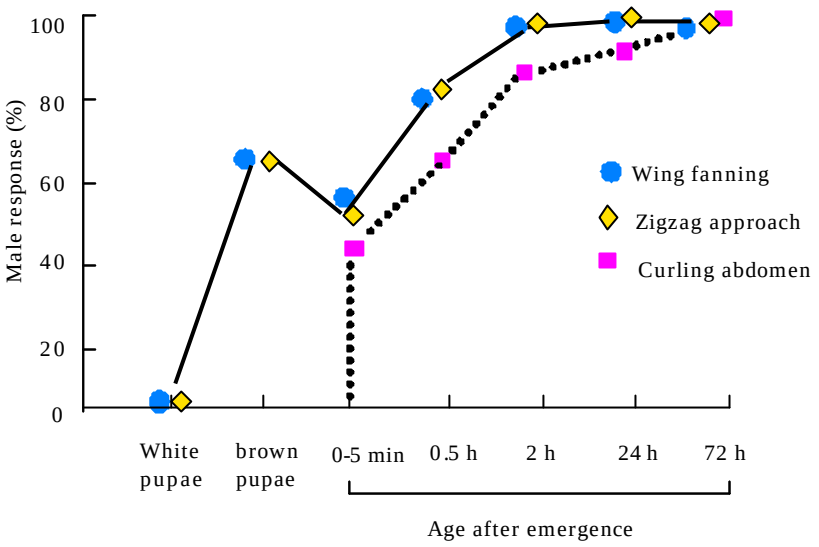
**Results**

Sexual maturity and responsiveness to sex pheromone

As shown in Fig. 3-2-2-a, sex pheromonal activity of females changed as their age increased. Immature female pupae of white color triggered no response in the male flies, but matured female pupae of brown color elicited WF and ZA responses in 63% males. These activities increased and reached a maximum 24 h after female emergence. No matter what stage the pupae was, female pupae elicited no CA responses in males. On the other hand, female adults in each stage of emergence elicited CA responses (Fig. 3-2-2-b). This activity reached 100%, 24 hours after

female emergence.

(a)




(b)

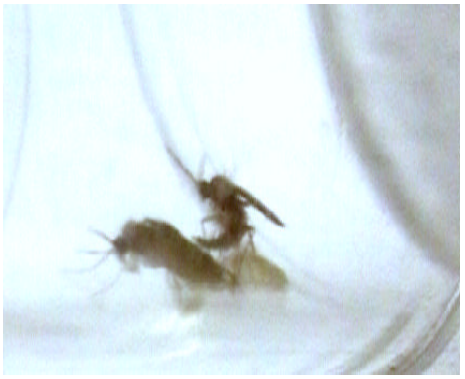


Fig. 3-2-2. Behavioral responses of male *B. paupera* to different ages of females in individual assay (a), and photograph of a male curling abdomen to copulate with an emerging female (b).

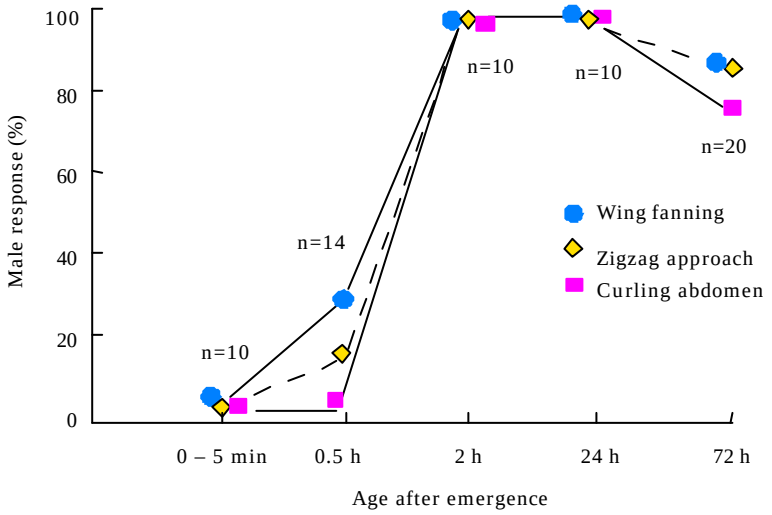



Fig. 3-2-3. Behavioral responses of different ages of *B. paupera* males to virgin females in an individual assay.

Males showed no response to females soon after emergence (Fig. 3-2-3). Even at 30 minutes after emergence, only 29% of the males responded with WF and ZA. However, WF and ZA and CA responses in males reached 100% 2 h after emergence, but the responses of males showed decreasing trend 72 hours after emergence.

Pheromonal activity of crude extract

Responses of males to different amounts of female extracts are shown in Fig. 3-2-4. Males were able to respond by WF and ZA to concentrations as low as $2 \times 10^{-3}$ FE, exhibiting high sensitivity to sex pheromone. The response of males showed an dose dependent increase to the female extracts. All males behaviorally responded when exposed to the 2 FE extract. However, CA behavior was not observed in males within the concentrations of extracts applied in this experiment.

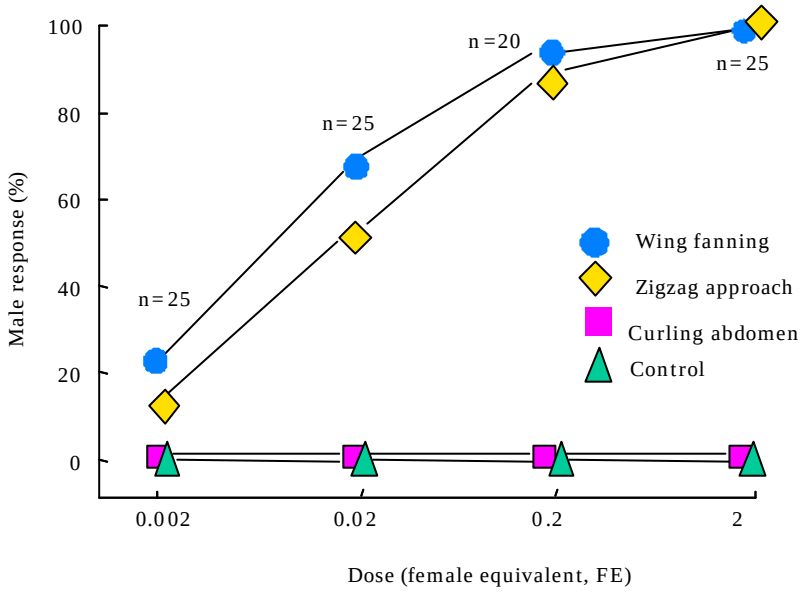



Fig. 3-2-4. Behavioral response of males to female extracts in an individual assay.

Persistence of pheromonal activity

After awakening from anesthesia (at -10℃ for 5 min), females showed complete pheromonal activity (Table 3-2-1). As the preservation period increased, female corpses elicited 80% for WF, 80% for ZA and 68% for CA in 2 h old males, and 80% for WF, 70% for ZA and 65% for CA in 3 day males, demonstrating persistence of the female sex pheromone. After 30 days, however, WF and ZA responses were obviously reduced to both 20%, and CA responses completely disappeared. Activity of female corpse preserved for 3 days under dried conditions showed a lower activity than frozen

preserved females for the same period, especially, CA responses were largely reduced.

Table 3-2-1. Behavioral responses of *B. paupera* males to female bodies with different treatments in an individual assay.

| Treatment (5 females) | No. of males | Response (%) | | |
|---|---|---|---|---|
| | | Wing fanning | Zigzag approach | Curling |
| Immobilized (5 min at -10 ℃) | 13 | 100 | 100 | 100 |
| Frozen for 2 h | 25 | 80 | 80 | 68 |
| Frozen for 3 d | 20 | 80 | 70 | 65 |
| Frozen for 30 d | 20 | 10 | 10 | 0 |
| Dried for 3 d | 25 | 60 | 60 | 20 |
| Dried for 6 d | 25 | 40 | 40 | 12 |

Location of female sex pheromone

Four body parts, head+ antennae, thorax+ legs, wings and abdomen and their corresponding extracts all showed sex pheromonal activity (Fig. 3-2-5). The abdomens showed comparatively higher activity, eliciting 68% for WF, 68% for ZA and 60% for CA responses in males. Corresponding abdominal extracts showed 100% for WF and ZA response in males. Head+ antenna were relatively low,

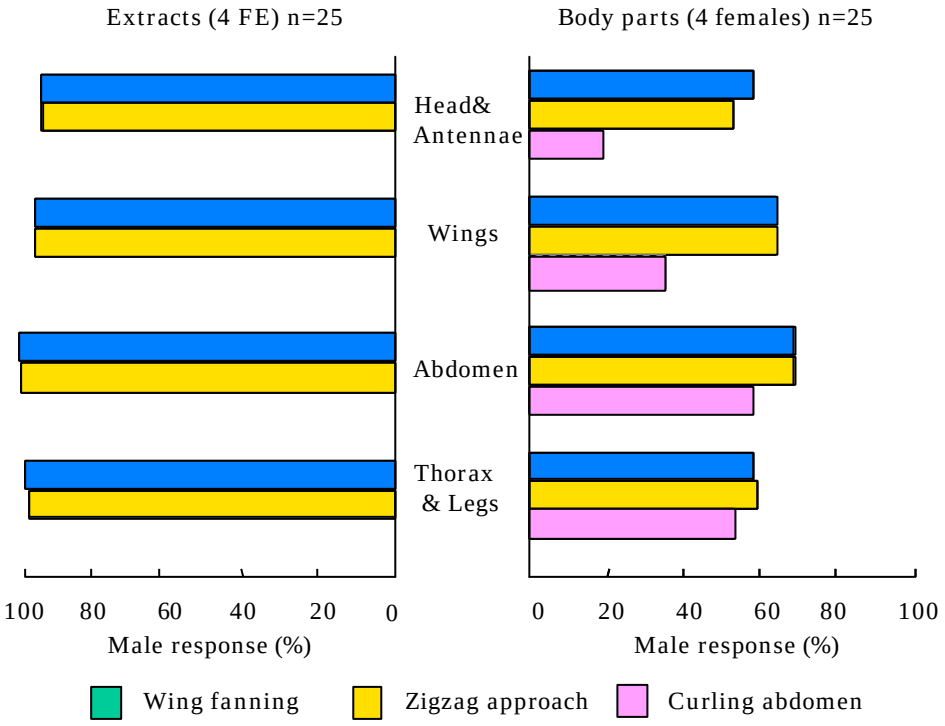



Fig. 3-2-5. Distribution of sex pheromones on the *B. paupera* female body.

eliciting 60% for WF, 56% for ZA and 20% for CA in males, and corresponding extract elicited 96% for WF and 96% for ZA.

Comparison of body parts and solvent extracts, showed the 4 FE extract elicited higher responses than the 4 body parts did. This difference may be due to the emitting rate of solvent resolved sex pheromone being more rapid than female body parts.

The above results suggest female sex pheromone is distributed throughout the female body with abdomen and thorax containing higher amounts than the other parts.

Factor for abdominal curling

Each tested male responded to the extracts of female wings with WF and CA but not CA responses similar to the results in all tests using female extracts. Female wings alone elicited 86.7% of WF, 80% of ZA and 73.3% of CA responses in males (Fig. 3-2-6), but males showed no response to male wings. When male wings

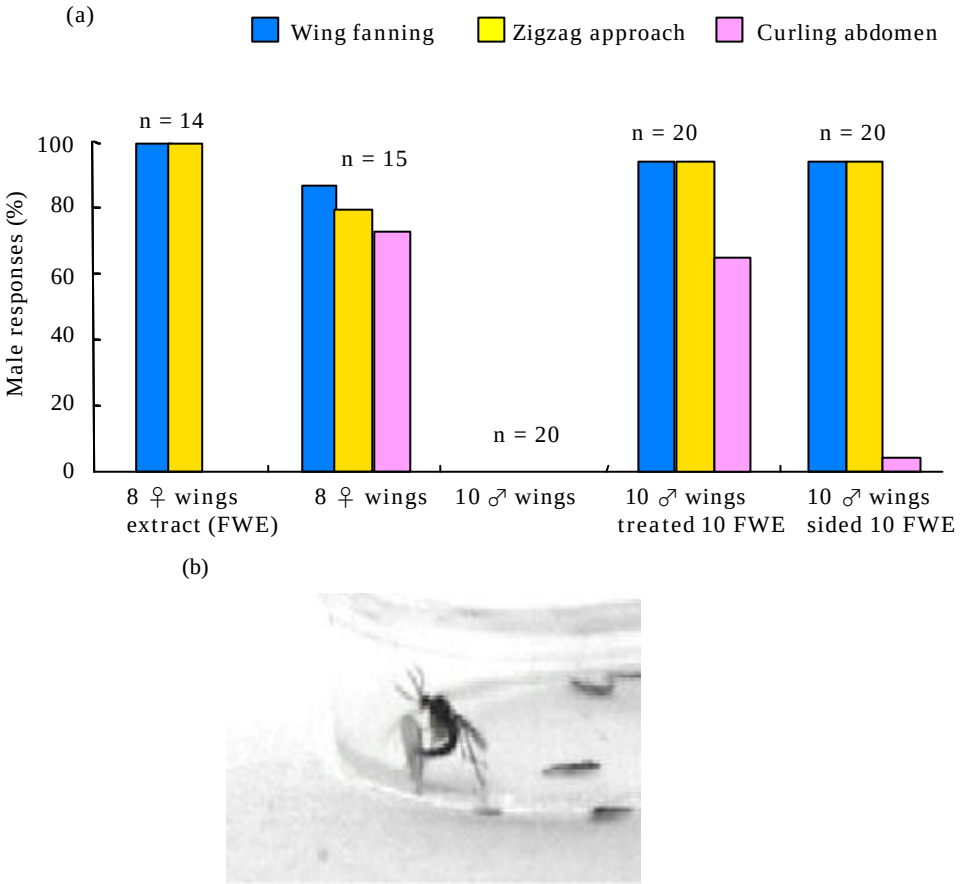



Fig. 3-2-6. Male *B. paupera* responding to wings and wing extracts of both sexes in an individual assay. (a) bioassay result, (b) photograph showing a male curling abdomen to a piece of female wing.

Table 3-2-2. Long distance attractability of male flies to female extracts of *B. paupera* in a wind tunnel test

| Time after male release | Treatment | Male flies (%) | | | | |
|---|---|---|---|---|---|---|
| | | Section-1 | Setction-2 | Setction-3 | Section-4 | Trap |
| 3 min | Test | 10.0 | 6.7 | 8.3 | 21.7 | 53.3 |
| | | * | n.s | n.s | n.s | * |
| | Control | 59.0 | 13.5 | 8.0 | 13.6 | 5.3 |
| 8 min | Test | 11.7 | 5.0 | 1.7 | 15.0 | 66.7 |
| | | n.s | n.s | * | n.s | * |
| | Control | 35.5 | 7.5 | 12.5 | 35.0 | 10.0 |

N= 20, 3 replicates for test, 2 replicates for control.

*Significantly different from control at P < 0.5 by Wilcoxon t-test. n.s :not significantly different.

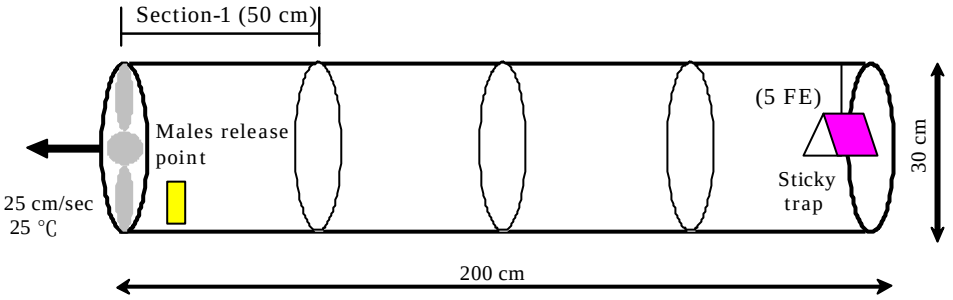



Fig. 3-2-7. Schematic of the wind tunnel used for attraction activity test.

were directly coated with extracts of female wings, the combination evoked a CA response of 65% in males, but when female wing extract was applied to a piece of filter paper and placed next to male wings, only 5% CA response was observed in males. This result demonstrates the factors eliciting male CA behavior seem not to be visual cues, because males responded not by discriminating the difference in the wing shapes of males and females visually. By comparing the results of placing FWE aside male wings and applying FWE directly on male wings, it appears males may also have factors that contribute to CA responses, which are not included in solvent extracts of females.

Distance attractive effect of the sex pheromone

In the wind tunnel test, 5 FE of crude female extract lured 53.3% of the released males to fly upwind and trapped in the sticky trap after 3 min (Table 3-2-2). The trapped males increased to 66.7% of the males released after 8 min. In the control test, only 5.3% of males flew into the trap at 3 min and 10% after 8 min, whereas 59.6% of the tested males stayed in section-1, obviously higher than 10%

of the 5 FE lure. Female crude extracts were shown to possess significant attractive effects on male flies.

**Discussion**

In this section, existence of a female sex pheromone is further supported by a series of bioassays. In addition female sex pheromone shown strong attractiveness to males in a wind tunnel, as shown in *B. impatiens* (Alberts et al., 1981). Sex pheromonal activity was detected throughout the female body, as shown in *L. mali* (Gotoh et al., 1999). Some results were very characteristic e.g. presence of sex pheromonal activity in the pupa stage and persistence of sex pheromone in corpses.

According to Sasagawa (1985), male *B. agrestis* when walk around searching for females in a copulation attempt appeared without discrimination, even tring to copulate with males or corpses. In the current experiment, similar behavior was also observed in *B. paupera*. The male *B. paupera* would try to re-copulate with females that they had just finished copulating with.

Female bodies began showing sex pheromonal activity at an earlier time at late pupa stage and maintained the effect after mating or even after death. Female flies seemed not to be able to control the secretion of sex pheromone as most lepidopteran insect with pheromone glands. This may related to the background of the mechanism of the production and secretion of sex pheromone in Dipteran species, that is still not fully understand. In Lepidopterans, sex pheromone biosynthesis is often mediated by a 33 or 34 amino acid pheromone biosynthesis activating neuropeptide (PBAN) through alteration of enzyme activities at one or more steps prior to or during fatty acid synthesis or during modification of the carbonyl group (Percy-Cunningham and MacDonald, 1987). But in Dipterans, it appears that ovarian-produced ecdysteroids regulate synthesis by affecting the activities of one or more fatty acyl-CoA elongation enzyme(s) during hydrocarbon sex pheromone biosynthesis. Hydrocarbon pheromones studied in model Diptera are synthesized in specialized subcuticular abdominal epidermal cells (oenocytes) and deposited onto the cuticular surface (Dillwith and Blomquist, 1982; Ismail and Kremer, 1983; Langley and Carlson, 1983). For example, the hydrocarbon pheromones synthesized in the abdominal oenocytes by the laboratory fruit fly, *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) (Coyne and Oyama, 1995; Ferveur et al., 1997), are transported by lipophorin (Pho et al., 1996) to epidermal cells for deposition on the cuticular surface. However, these results still need to be clarified. If we assume female *B. paupera* also synthesize sex pheromones in this way, this may explain the phenomena that sex pheromone

activity is present at the pupae stage and persistency until death, because cuticular wax substances may continually be synthesized from early in the life of the imago and eluted the duration of the fly life from oenocytes in the epidermis (Ferveur et al., 1997).

The matured female pupae triggered WF and ZA responses in the male adults, but only the emerged female adults or female extracts applied to the body parts e.g. wings elicited CA responses in male adults, with out combination either the shape and color nor extracts loading on filter paper elicited CA responses. This suggests that factors triggering WF or ZA responses and CA responses are different. However, since sex pheromonal activity of female extract especially the attractiveness which was centered concerning of this study appeared accompanying with WF and ZA, further elucidation of CA factor were not conducted.

Sex pheromones of this species expressed strong attractive properties when female extract were tested in a wind tunnel. The male flies showed an ambiguously chemotaxis flight and correct landing on the stimuli source.

The aggregation pheromone have been identified in some species, as in *Drosophila hydei* (Moats et al., 1987), *D. martensis* (Schaner and Jackson, 1992), *D. serido* (Schaner and Jackson, 1992), and some sex pheromone were practically used, as in olive fly, *Dacus oleae* (Jones et al., 1983) and in *Toxotrypana curvicauda* (Landolt et al., 1988).

Based on the above results, the sex pheromone of this species appears to possess potential for development of management options.

## Section 3. Effect of light on chemotaxis to the sex pheromone

### Materials and Methods

Mating behavior under dark

To investigate the effects of light and dark on mating behavior in the *B. paupera*, 30 pairs of virgin females and unmated males were separately observed under a red lamp (1 lx) in the glass tube as described in section-1 (Observation of mating behavior). Mating behavioral components WF, ZA, CA, P180 and MO were recorded the same as before, and analyzed for significant differences between the light and dark condition by Mann-Whitney U-test at $P<0.05$.

Light intensity and attractivity of sex pheromone

To investigate the effects of light intensity on the chemotaxis of males to female sex pheromone in a wind tunnel, the same wind tunnel apparatus described

in section 2: wind tunnel test (Fig. 3-2-7) was employed. A series of light intensities were produced by installing a metal roof above the wind tunnel and lifting or lowering the metal roof which hold a fluorescent lamp (40 W HITACHI), or by wrapping black net (DAIHO Chem. Co. LTD) and black plastic film (SEKISUI Chem. Co. LTD) around the lamp. The light was graded to 8 intensities, 1500 lx, 1000 lx, 500 lx, 100 lx, 50 lx, 10 lx and 1 lx (dark, red lamp). The intensities of the light in the wind tunnel were measured by a digital illuminometer (T-1, MINOLTA Co. LTD) at 3 places (male release point, lure point and in the middle) prior to the start of each test. In each of these 8 graded illuminations, the wind tunnel test was replicated 3 times per release of 20 male flies. All tests were conducted at 25 ± 2℃, 60-70% humidity and a flow velocity of 25 cm/sec. The results were statistically analyzed for significant differences by Mann-Whitney U-test with Bonferroni correction at $P<0.05$.

Group assay under dark condition

The same apparatus described above was employed for the group assay. Responses of 5 male flies to 10 FE and 20 FE were observed under a red lamp (ca. 1 lx), and a red lamp plus fluorescent light (500 lx). The light intensity was measured and verified by a digital illuminometer (T-1, MINOLTA Co. LTD). Each combination was replicated 4 times. The response rate of landing on the sex pheromone source (LO) and duration of flight from take off to landing was measured and recorded. Significant differences were analyzed by Wilcoxon t-test at 0.05 level.

Comparison of the motility of male flies in light and darkness

A cage (used in the group assay) containing 20 males randomly caught from the massive rearing box was placed in front of a video camera (DCR-TRV-310, SONY) (Fig.3-3-4a). After males adapted for about 30 min, the movement of the flies was recorded continuously with the video camera under a photo regime of L5:D5 min to more than 30 min. Light to dark changes were accomplished by switching the power of the red lamp (Dark, 1 lx) and fluorescent lamp (Light, 500 lx) installed above the cage. With another replication, both light and dark condition were recorded to total of 3 × 2 times and 5 × 6 min. The videos were transferred to a digital file format by connecting video recorder to a computer installed of Windows XP (Microsoft) operating system. The video files were cut to 5 min length video clips by using application software (Moviemaker, Microsoft).

Analysis of motility of the males was conducted within the time from 2 min to 4 min for each 5 min clip by counting the number of flying or walking males within 2 sec scenes when passed every 5 seconds while replaying the video clips. The difference in the motility of the males between dark and light conditions was

analyzed for significance by paired t-test.

## Results

Effect of darkness on mating behavior

Under darkness, the male flies could perform the 5 mating behavioral

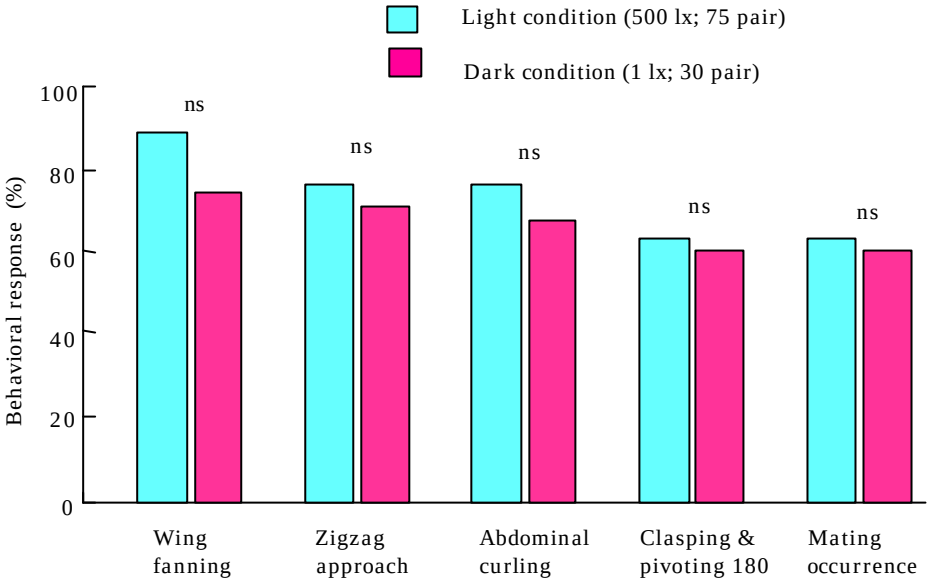



Fig. 3-3-1. Comparison of mating behaviors of *B. paupera* between light and dark conditions.

ns: not significant difference at P< 0.05 by Mann-Whitney U-test.

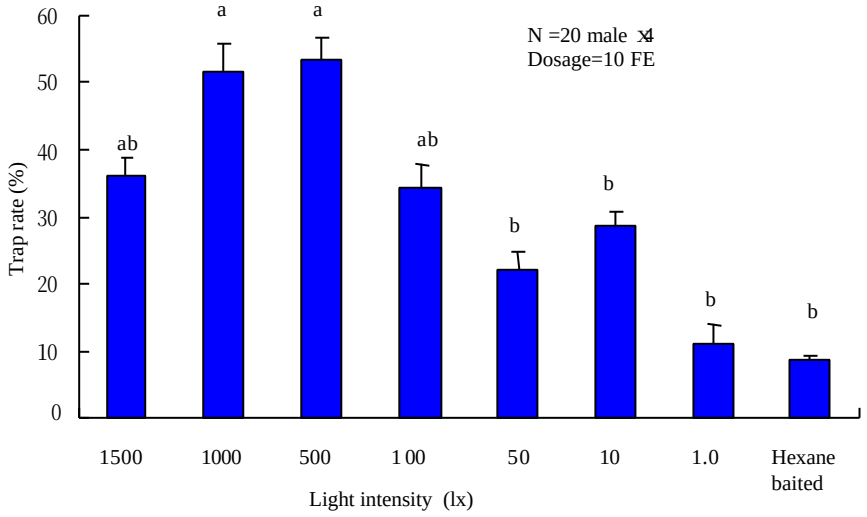



Fig. 3-3-2. Trap rate of male *B. paupera* lured by 10 FE in a wind tunnel test under different light intensities.

*Different letters indicate significant difference at P< 0.05 by Mann-Whitney U-test with Bonferroni correction.

components, WF, ZA, CA, P180 and MO normally at almost the same rate and sequences as observed under 500 lx (Fig. 3-3-1). In the dark males exhibited 73% WF, 70% ZA, 66%CA, 60% P180 and 60% MO in sequence, while in the 500 lx light condition the rates were 87.5% WF, 75% ZA, 75% CA, 62.5% P180 and 62.5% MO. These results show the mating behavior of the fly under dark conditions is not obviously affected by illumination.

Effect of light intensity on trap rate

Rate of trap catches under different light intensities in the wind tunnel lured with 10 FE crude female extract is shown in Fig. 3-3-2. In a series of light intensities, the landing response of males was comparatively high under 500 and 1000 lx at 51.9% and 53.4 %, respectively. However, as light intensity decreased dim, sex pheromone trap catches decreased with the decreasing illumination to 28.5 % under 10 lx and 8.84% under 1 lx. Significant differences were observed between illuminations above 500 lx and lower than 50 lx. Rate of trap catches under1500 lx and 100 lx were observed between the two different groups. This shows illuminations lower or higher than 500-1000 lx affected the trap rate.

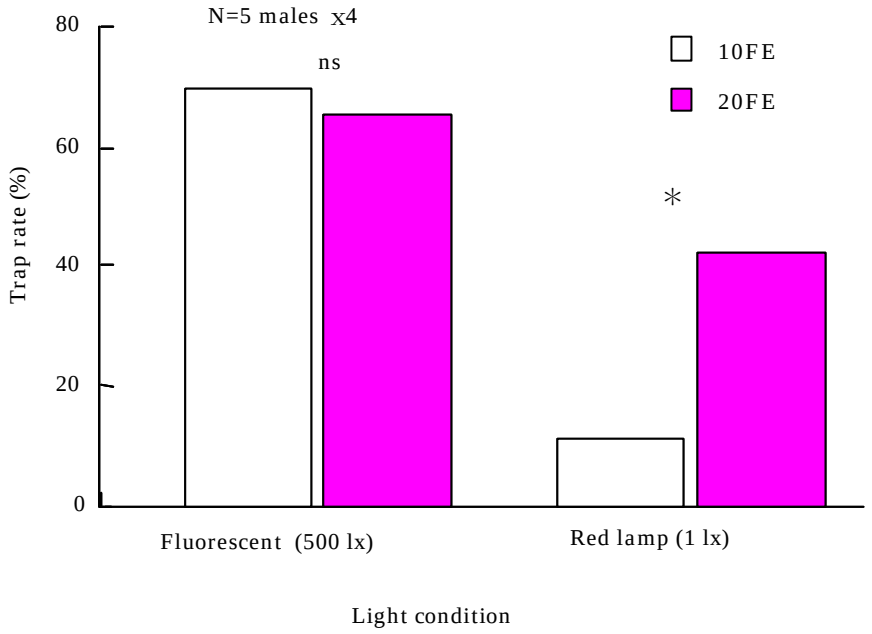



Fig. 3-3-3. Trap rate of male flies lured by 10 FE and 20 FE of female extracts under light (500 lx) and dark (1 lx) conditions in the group test.

* Significant and not significant difference (ns) at $P < 0.05$ by Wilcoxon t-test.

Effect of light and dark on the group assay

In the group assay, the response rate of LO decreased to 10% when males were

exposed 10 FE female extract under red lamp (1 lx),, while under a fluorescent light (500 lx) the rate was 70% (Fig. 3-3-3). When the amount of sex pheromone was increased to 20 FE of the stimulus source, the number of males catches rose to 44% under the red lamp and 65% under light conditions. A significant difference was obtained between dark and light and between 10 FE and 20 FE in the dark conditions. These results show that increasing the sex pheromone dosage may have a complementary effect, but can not counteract the effect of light conditions.

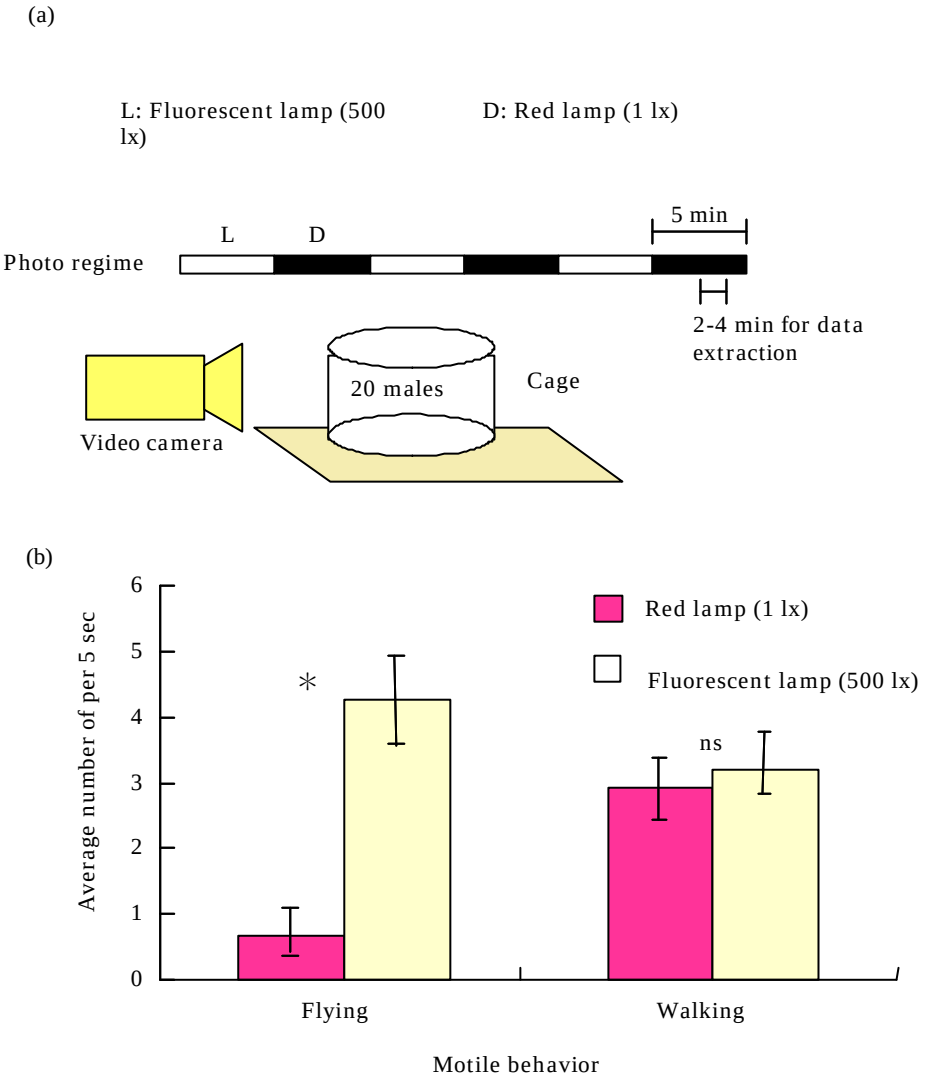



Fig. 3-3-4. Comparison of motility of male flies under light (500 lx) and dark (1 lx) conditions. (a) Schematic of the experimental method, (b) behavioral statistical analysis results.

* Significant and not significant difference (ns) at $P < 0.05$ by paired t-test.

Comparison of motility of male flies under light and dark

The males showed a distinct difference in flight behavior between light and

dark conditions. In the dark condition, the mean numbers of flying males during each 5 sec scene within an interval of about 2 sec was 0.53 ± 0.36 (Fig. 3-3-4-b) and the number of walking males was 2.98 ± 0.47, while under the fluorescent lamp the average number of flying males was 3.79 ± 0.69 and walking was 3.05 ± 0.41. Obviously the flying behavior of the males was sharply depressed by dark conditions, but the walking behavior remained at the same motive level.

**Discussion**

In the experiment covered in this section, paired male and female *B. paupera* were able to complete the stereotyped mating behaviors in both light and dark conditions in a glass tube. No dial periodicity has been reported in sciarid flies (Sasagawa, 1985; Gotoh et al., 1999), thus sexual communication and accession of females by males flies at close range appears to be affected by lighting condition.

In the wind tunnel, light intensities higher than 1500 lx or lower than 500 lx both showed decrease in numbers of the male *B. paupera* trapped. The effect of light intensity on chemotactic behavior may be a result of the locomotor activity being depressed by high or low illumination. The effect of illumination intensity on insect behaviour is well reported in the literature. A light of a particular intensity can trigger or inhibit sexual behaviour of some insect species (Shorey, 1974) and can also influence the speed of locomotion and activity of various species of invertebrates. With a series of two-choice tests, Rakowaski (1988) reported that illumination intensity can influence the response of adult hide beetles, *Dermestes maculates*, to aggregation pheromones by decreasing the locomotor activity of beetles in dim lights (1 and 10 lx). And increase in light intensity also resulted in a diminishing effect on the aggregation pheromone under high illumination (over 10,000 lx). Flight behavior of the male *B. paupera* decreased obviously when switching to dark conditions, but walking behavior was not significantly different. This indicates all the locomotor activities were not sharply changed within the 5 min. A decline in the number in male *B. paupera* caught in the wind tunnel was likely due to suppression of flight activity by low light intensity.

A decrease in flight activity may be due to loss of optical information vital for the fly flight. In a simple test (not included result), tweezers were moved rapidly towards a resting fly under light conditions, the fly quickly fled away, but under the red lamp conditions the fly did not respond to the danger. Obviously in the red lamp conditions the tweezers were invisible to the fly. The fly can also react to visual stimulus, despite indisputable important roles of chemical cues as was demonstrated in the last section.

Generally most of dipteran insects are diurnal animals in nature before greenhouse dwelling began. Fungus gnats inhabit moist shady areas within woodlands and field crops (Harris et al., 1996). Thus it is possible that sciarid flies have developed a proper light range for living.

According to Gotoh et al. (1999), mushroom greenhouses operate in a regime of dark except during working time, but in recent years often fluorescent black lamps are used as control procedures. Although Jess and Kilpatrick (2000) reported that light-trapping can stimulate fly activity and immature stages in the growing substrate, no detailed evidence has been given. Lights-on may be a minus for mushroom growth (Ishitani, 1997), but black lamps remain the only practical application in mushroom houses.

In the last section, the feasibility of utilizing the sex pheromone of this species for development of control measures is possible, because the pheromone demonstrated strong attractiveness. The results of this section prompted me to consider the effect of illumination on pheromone application in order to design a better attractant sex pheromone trapping method with the proper fluorescent lamp.

## Chapter Ⅳ. Modification of EAD and Monitoring Bioactive Component

In the last chapter chemical cues were demonstrated to actually be involved in the sexual communication of *B. paupera.* Female extracts appeared to function as key factors regulating mating behavior and effective attractants of male flies. Thus I became encouraged to isolate the active components and elucidate their chemical structures, and further to examine the feasibility of manipulating insect behavior with laboratory synthesized chemicals.

In order to determine candidate sex pheromone components, effective methods for sampling, extracting, separating and further tracking of the most active components need to be experimentally developed and employed according to the secretory characteristics of the sex pheromone of the targeted insect (Stevens, 1998). The chemical composition of female *B. paupera* extract was expected to be complex because the extract came form washing the whole bodies of females with hexane. Thus preliminary separation utilizing common techniques was essential to the pheromone identification.

Most separation methods for crude extracts usually involve one or more forms of chromatography. Liquid chromatographic methods are traditional but some what of samples and time consuming (Heath and Humlinson, 1984) because it requires

concentration and separate testing of each fraction. Also using large volumes of solvents can bring impurities into the sample. Thin layer chromatography (TLC) provides a rapid and easy method for evaluating a number of possible solvent systems for the separation of a mixture and also provides a useful way of following the progress of a column chromatogram.

In recent years the coupling of GC and electroantennogram (EAG) fully utilizes the tremendous analytical capabilities of these two techniques leading to the development of extremely sensitive and specific detection systems for pheromone components. A extracted mixture can be screened rapidly without the necessity for fractionization and separate testing of all fractions (Hows, 1998). The relative structure-activity relationships of a homologous series of compounds can be readily determined by gas chromatography electroantennographic detector GC-EAD (Struble and Arn, 1984). GC-EAD takes full advantage of excellent separation and detection capabilities is almost indispensable for identification of active compounds (Malo et al., 2000). The utilization of the advantages of GC-EAD seemed necessary for the identification of sex pheromone in *B. paupera*.

However, EAD responses from very small insects are difficult to discern from background noise (Roelofs, 1984). This has been largest factor preventing the identification of pheromone of small sized insects like sciarid flies.

Moore (1981) reported a technique to amplify the EAG signals based on an in series resistor, capacity theory and the resulting sound signals, This technique appeared worthy to try on this species.

EAD responses however can be a poor indicator of behavioral responsiveness because depolarization depends upon the number of neurons stimulated, does not distinguish between neurons that may have inhibitory or synergic effects at various integrative levels and a small number of neurons may control a key response.

In this chapter preliminary separation of the crude extract was conducted by TLC and column chromatography (Section 1), then modification of the GC-EAD (Section 2) allowed recording of the fly's olfactory responses to the contents of female and male crude extracts (Section 3).

## Section 1. Preliminary separation

### Materials and Methods

Thin layer chromatography

A TLC plate (20 cm ×20 cm, silica gel 60 F250 MERCK) activated at 100℃

for 6 hr in a oven, was spotted on with the crude female extracts (ca. 1600 FE), then set in a glass vessel (12.7 cm ×25 cm ×25 cm) and developed with 5% ether in hexane (e/h). The position of solvent uploaded components were visualized by placing the plate in an iodine vaporing vessel for 5 min. Each 2 cm of the horizontal zones was scrapped off and triply rinsed with hexane to give 9 fractions (Fig. 4-1-1-a). The sex pheromonal bioactivity of 9 fractions (Fr.) was examined by the individual assay at a 16 FE dosage. The effluent of active fraction was preserved at – 20℃ for later identification use.

Silica gel chromatography

The crude extract of 38170 FE females was chromatographed on 30 g of silica gel (Wako gel C-200, Wako), pre-activated at 100 ℃ for 6 hr, places into a column (Hi. 30 cm × id. 1 cm). The crude extract was eluted successively with hexane (50 ml + 100 ml), diethyl e/h 5 % (100 ml + 100 ml + 50 ml), 10 % (50ml + 100 ml), 50% (300 ml) then MeOH in diethyl ether 50% (150 ml). Each fraction was concentrated to 2 ml and checked pheromonal activity by individual assay with 16 FE.

The contents of each fraction was monitored by GC (GC-17A, Shimazu) with DB-1HT (J&W) column at a temperature temperatures program of 50℃ for 2 min, to 250℃ at 10℃/min for 20 min.

Preparative capillary gas chromatography

Preparative capillary gas chromatography was performed by GC (6890N Network System, Agilent Tech.), with a HP-5 glass capillary column (30 m x 0.25 mm ID) coupled to a preparative fraction collector (PFC, Gerstel Co. LTD). The temperature was programmed after two preliminary injections from 40℃ to 61℃ at 3℃/min after 1 min for sampling time, 61 to 240℃ at 10℃/min and then held for 24 min. Helium was used as the carrier gas at a constant flow rate of 35 cm/sec. The GC effluent was split at a ratio of 1:100 between the FID and to the collector. Temperature of the collecting device and transfer-line were 240℃. Collector trap no. 1 to no. 4 were at - 10℃ and trap 5 at ambient. Crude extracts samples were injected into the column by a cooled injection system (CIS-4, Gerstel Co. LTD).

Crude extracts of 300 female equivalents (FE) were injected (3×100 FE /5 μl) and automatically separated into five fractions according to retention times (Rt): Rt 0 - 4 min for Fr - 1; Rt 4 -16.5 min for Fr - 2; Rt 16.5 - 23 min for Fr - 3; Rt 23 - 26 min Fr - 4; and Rt 26 - 50 min for Fr - 5. The design of fraction cutting of the crude extracts is shown in Fig. 4-1-4-a. The recovery rate of fractions was calibrated to ca. 50% by injected standard hydrocarbons C15 to C31 under the same analytical conditions. The bioactivity of each fraction and their combined effects was determined by the group assay at 10 FE per test.

## Results

Thin layer chromatography

On the TLC plate 4 bands from the base to the front of a 18 cm high run area,

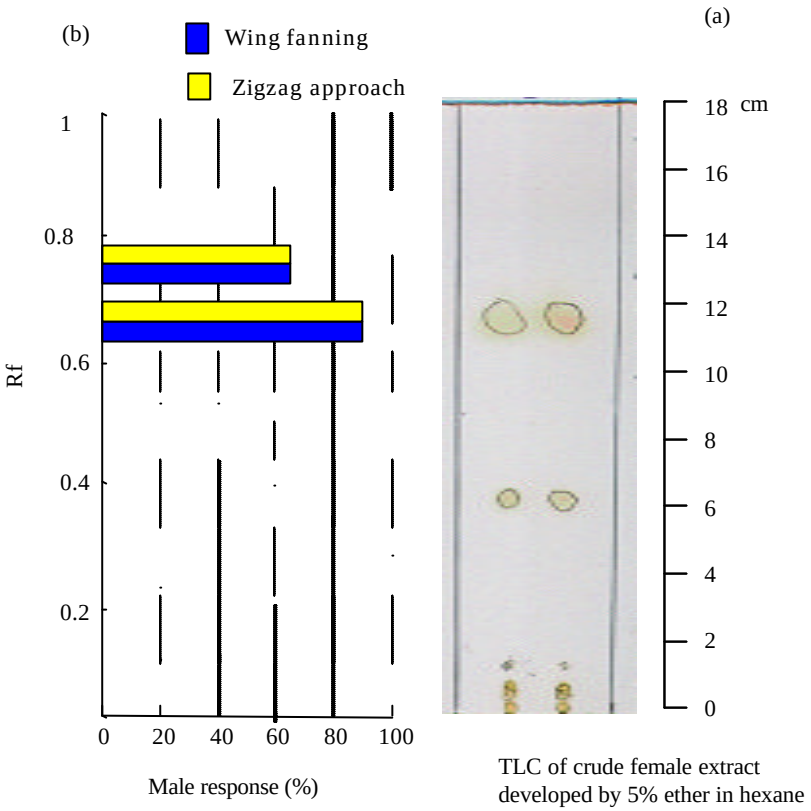



Fig. 4-1-1. Pheromonal activity of TLC fractions checked by the individual assay.

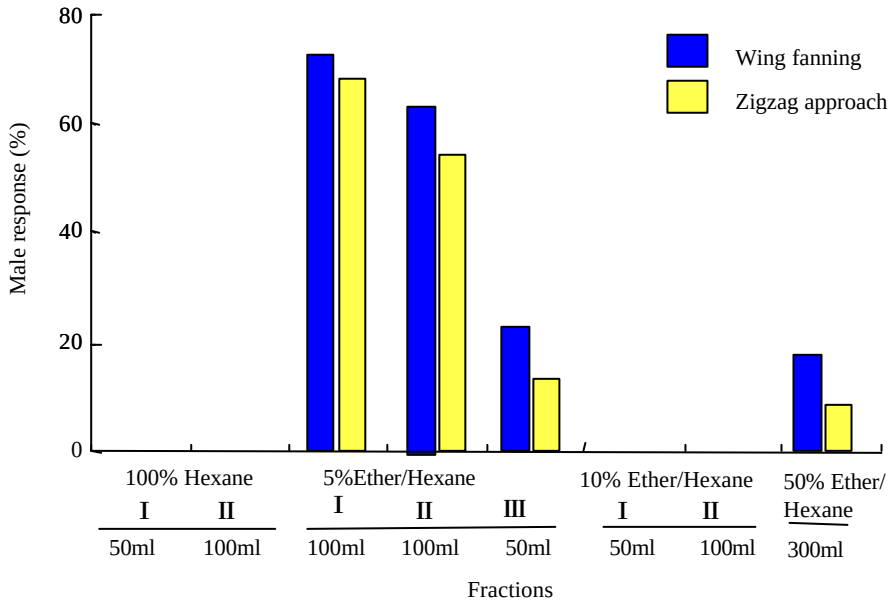



Fig. 4-1-2 Pheromonal activity of fractions of crude female extracts separated by silica gel chromatography in the individual assay.

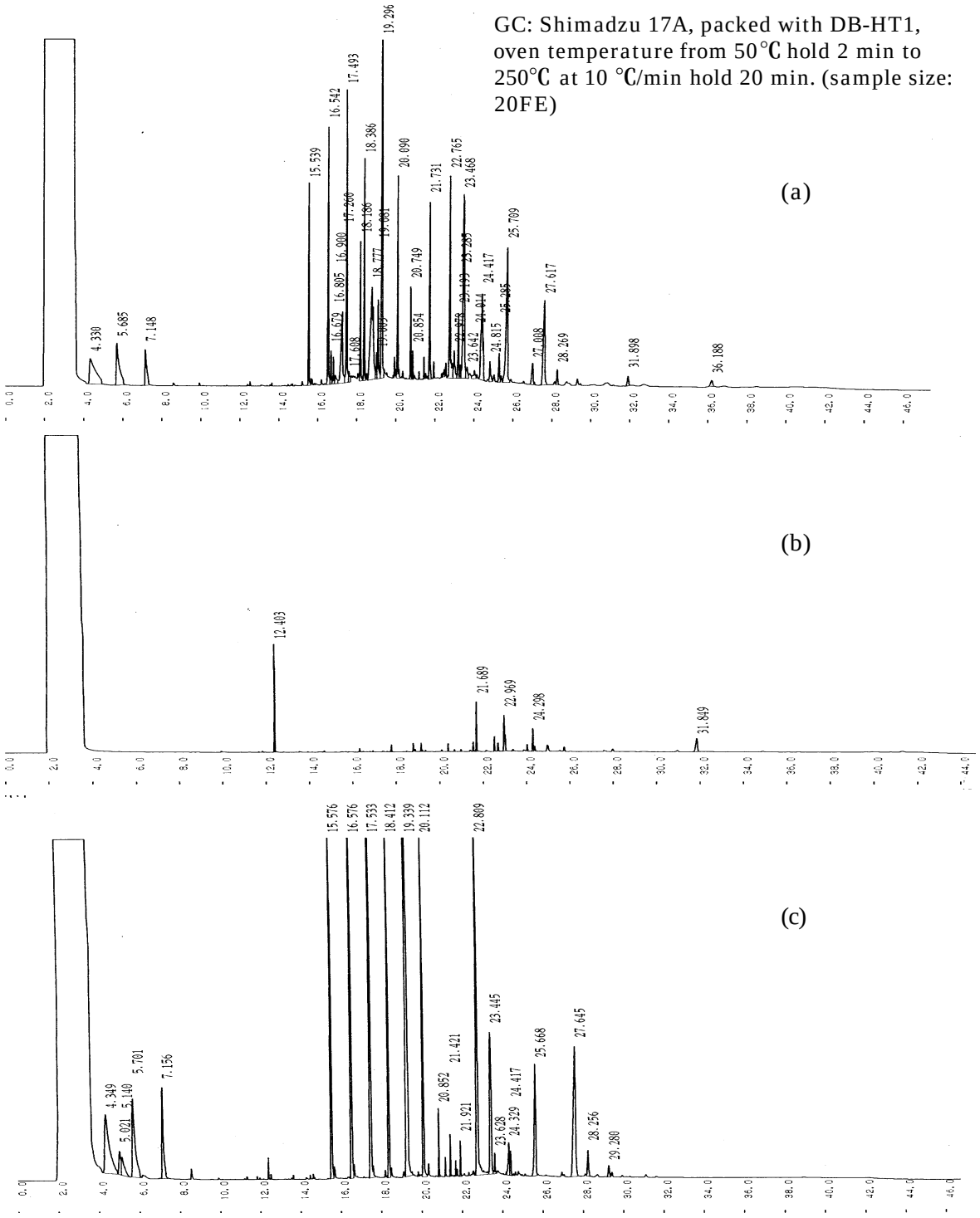



Fig. 4-1-3. Gas chromatogram of female crude extract (a), 5% ether/hexane-1 fraction (b), and 50% ether/hexane fraction (c).

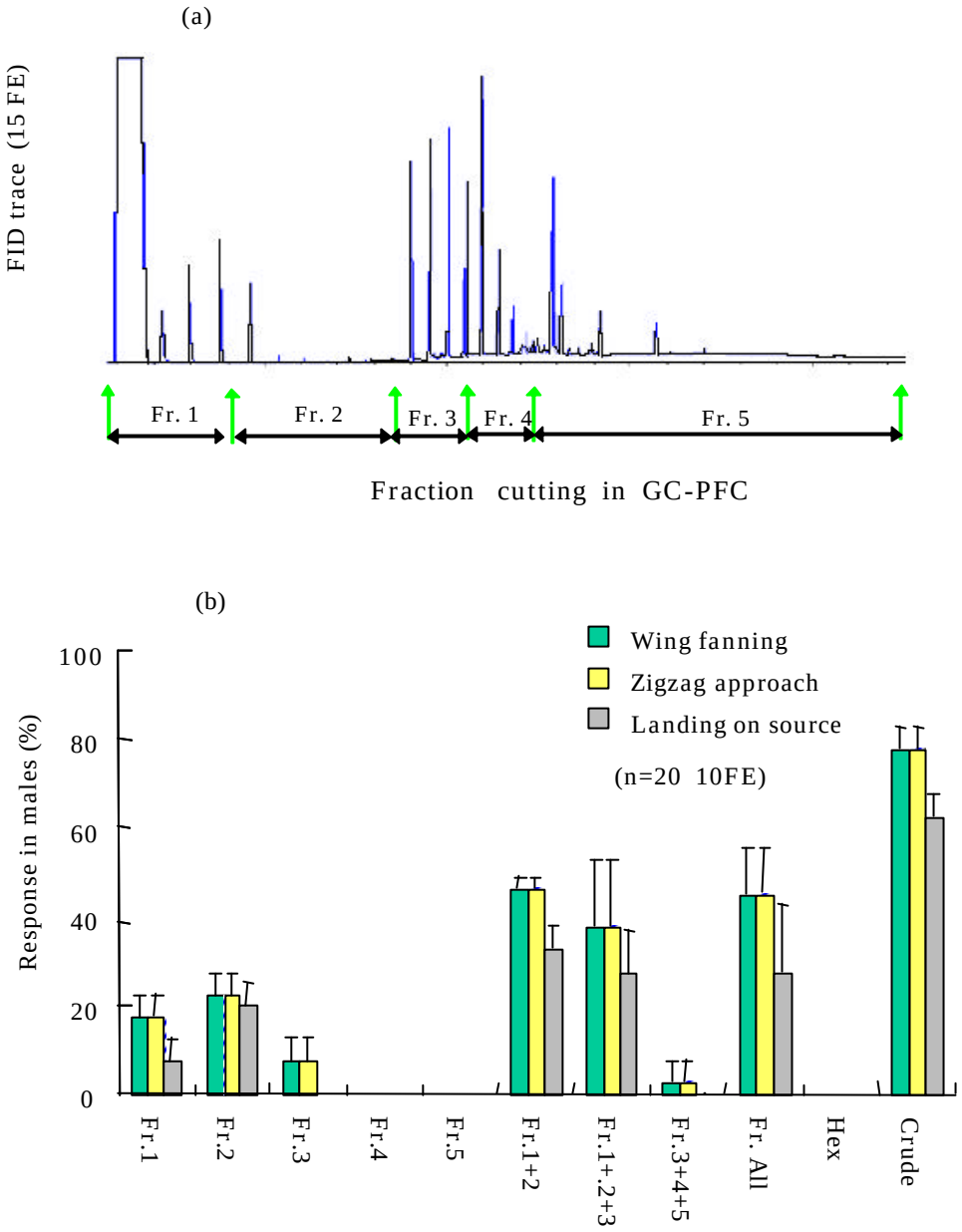



Fig. 4-1-4. Schematic of designative cutting of fractions in GC-PFC (a), and pheromonal activity of GC-PFC fractions of crude female extracts in the group assay (b).

became visible after iodine fumigating (Fig. 4-1-1-b). The individual assay showed extracts from 10-12 cm and 12-14 cm evoked behavioral responses in males of 90% WF and 90% ZA and 65% WF and 65% ZA, respectively. Other fractions showed no response by males. The Rf (rate of flow) values of the 10-12 cm fraction and 12-14 cm fractions were between 0.62 – 0.78.

Silica gel chromatography

Eight fractions from silica gel chromatography were assessed of sex pheromonal activity by the individual assay. The active components were mostly eluted by5 % e/h and elicited 73% WF and 68% ZA; 64% WF and 54% ZA ; 23% WF and 14% ZA in Fr. -1, Fr.-2 and Fr.-3, respectively. The 50% e/h fraction was less active evoking 18% WF and 9% ZA in males. The 100% hexane Fr., 10% e/h Fr. and 50% methanol in ether (m/e) Fr. were not active (Fig. 4-1-2).

The GC of the crude extract, 5% e/h Fr.-1 and 50% e/h fraction of the silica gel chromatography are shown in Fig. 4-1-3. The representative cuticular substances appear to be present in the 50% e/h fraction with just a small amount in the 5% e/h fraction.

Activity of GC-PFC Fractions

In fractions collected by GC-PFC (Fig. 4-1-4-b), fraction 1 elicited 17.5% WF, 17.5% ZA, and 7.5% LO response in males. Fraction 2 evoked 22.5% WF, 22.5% ZA, and 12.5% LO and fraction 3 evoked 7.5% WF and 7.5% ZA in males. No fractions behind the 26 min retention time or control showed activity. Increased responses in WF of 46.25%, ZA of 46.25% and LO of 32.5% equal to half the effectiveness of the crude extract was observed by a mixture of Fr. 1 and Fr. 2, but no additive effect or synergistic effect was seen when combined. The results indicate the domain of the sex pheromone is in Fr. 2 and Fr. 1, despite comparatively low response percentages.

**Discussion**

The active compounds were mainly positioned at Rf 0.67 (TLC) when the crude extract was developed by 5% e/h solvent. Thus, the 5% e/h solvent was thought to dissolve most of the sex pheromone active components. A single band evoked effective pheromonal activity, means the active substance may be consisted by chemicals of similar polarity. Furthermore, it was able the active substance appeared to be weak polar chemicals, likely branched hydrocarbons, ethers, esters, terpenes and aldehydes (Suzuki et al., 1990).

This assessment agreed with the silica gel chromatographic separation in which the sex pheromone activity was almost complicit in eluted by 5% e/h solvent. However, the eluents did not contain a high profile of representative lipids. Comparison of the gas chromatogram of 5% e/h Fr.-1 and 50% e/h fraction indicated use active component(s) were low in concentration. Furthermore, fractions collected by preparative gas chromatography (GC-PFC) showed pheromonal activity mainly in fraction 2, but was a very low concentratio according to GC total ion

chromatography (TIC) of the female crude extract. This suggests the active component(s) are probably present as trace amounts in the extracts.

The results of TLC, silica gel column chromatography and GC-PFC separations suggest the necessity to utilize GC-EAD techniques to aid monitoring of the active substances, because it is difficult to determine candidates of the active components form the trace amount of lipids after step by step separation.

## Section 2. Selection of the shape and saline solution for GC-EAD

### Materials and Methods

The function of the antenna

Sixty males were captured from the mass culturing box and 20 males anesthetized at a time at -10 ℃ for 30 sec. Antennae were cut off of 20 males from the scape root, 20 males from half length of the flagellum and 20 males left intact. The response of these 60 males to 16 FE of female extract were determined by the individual assay.

Scanning electron microscopy (SEM)

Antennae of 2 females and 2 males were removed from the head and fixed on a brass holder with two-sided sticky tape. The antennae were coated with 6 nm platinum-palladium in a high-vacuum sputtering device (IB-3, EIKO Co. LTD). The surface structure of the preparations was observed by S-4200 SEM (HITACHI). Photographs of the ventral sides of the whole antennae were taken at a 470X magnification. The lengths and width of the male and female antennae were determined by referencing an inner scale label.

Comparison of 4 antennae linked in series to a single antenna bridge

Because the antenna of the *B. paupera* are very small (0.8-1 mm) it is difficult to prevent swallowing of the antenna by the surface tension of the saline solution when bridged between traditional chloridized silver electrodes. To determine a proper electrode shape model, 4 different shapes of antennal preparation models were made.

Model-A consisted of 4 antennae knotted together with a denier conductive gel (PARKE LABORATORIES INC) along a Hematocrit capillary glass tube (id. 0.2 mm × ed. 0.5 mm × L. 1 mm NICHIDEN-RIKA Glass Co. LTD) surface. Conductive gel was spotted at the antennae joints as an abutment on the glass tube. The glass tube was attached to a paired glass tube holder by double-sided sticky tape.

Model-B consisted of 4 antennae connected linearly by placing them on 3 pits

filled with saline solution. Three mini neighboring flutes grooved on a small acrylic plate polished by grinding were filled with conductive gel or 0.1 M KCl solution. The antennae was attached to the paired glass tube holder with double-sided sticky tape.

Model-C consisted of 4 antennae linked by lined capillary cuts filled with conductive gel. Four cut (L, 0.5 mm) Hematocrit capillary glass tubes (id. 0.2 mm × ed. 0.5 mm × L. 0.5 mm NICHIDEN-RIKA Glass Co. LTD) were polished and filled with the conductive gel or 0.1 M KCl solution. The antennae were also attached to the paired glass tube holder by double-sided sticky tape.

Model-D (Fig. 4-2-3) consisted of a single antenna bridged between two capillary glass tubes filled with saline solution. One antenna was set directly between paired glass tubes (interval ca. 0.5 – 0.9 mm).

The glass holder for holding the antenna preparation model was made from a pair of Hematocrit capillary glass tubes (id. 0.85 mm × ed. 1.65 mm × L. 75 mm NICHIDEN-RIKA Glass Co. LTD). The paired capillary glass tubes served as an electrode filled with 0.1 M KCl saline solution. The Ag wire (Dia. 0.5 mm) wes inserted into each electrode.

The responses of a set of the 4 antenna preparation models were checked sequentially on the sample eluting port of a coupled GC-EAD which employed basically the same techniques described by Struble and Arn (1984) with a slight modification. The GC was HP5890 II Packed with a nonpolar DB-1HT column (J&W Germen) and a temperature program was from 50℃ to 250℃ at 10℃/min then held for 20 min. The injection and detection port were at 250℃. The antennal response signals were transferred from saline solution by the Ag wire to a probe (JZ-802J, NIHONKODEN Co.), then amplified and filtered through a micro-electrode amplifier (MEZ-8201, NIHONKODEN Co.) followed by a Dual-beam memory oscilloscope (VC-10, NIHONKODEN Co.). The signals were finally recorded with a dual channel pen recorder (LR4420 CH1:range 100 uv, span -50:50, filter 0.1 Hz. CH2: range 2 mv, span -0.954:0.0425, filter 1 Hz) and monitored by a computer (IBM) with a AxoScope 8.2 (Axon Instruments, Inc. US) by an analogue signal input from a digitizer model.

Comparison of saline solution

Applicability of 6 compositions (Table 4-2-1) of saline solutions was tested by the above mentioned GC-EAD method. The composition of saline 3 were made according to Kaissling (1980). Saline 4 and 5 were made by increasing or decreasing some components of Saline 3. Saline 1 was a common solution used for lepidopteran (Roelofs, 1984) and saline 2 was made by adding glucose and NaCl to saline 1.

Solution 6 used in *Drosophila* sp neural physiological studies was made according to personal communication with Dr. M. Ozaki (Kyoto Inst. Technol.).

Table 4-2-1. Composition of 6 kinds of saline solution for GC-EAD recordings.

| | Saline-1 | Saline-2 | Saline-3 | Saline-4 | Saline-5 | Saline-6 |
|---|---|---|---|---|---|---|
| KCl | 740 | 740 | 1272.06 | 636.03 | 1272.10 | 61.42 |
| $KH_2PO_4$ | | | 125.12 | 250.24 | 125.12 | |
| $K_2HPO_4$ | | | 187.92 | 375.84 | 187.92 | |
| $MgCl_2$ | | | 0.09 | 0.05 | | |
| $CaCl_2$ | | | 11.09 | 5.55 | | 12.21 |
| KOH | | | | | | |
| HCl | | | 0.02 | 0.01 | | |
| $NaH_2PO_4$ | | | | | | 4.8 |
| $Na_2HPO_4$ | | | | | | 50 |
| Glucose | | 180.16 | 405.36 | 202.68 | 405.36 | |
| NaCl | | 58.44 | 146.10 | 73.05 | 146.10 | 649.85 |

(mg/100 ml)

After the antenna preparation was set on the sample port, 5 FE of crude female extract as stimuli was given at 1, 5, 10, 15, 20, 30, 40, 50 and 60 min by puffing 1 ml sample into the glass duct by a syringe sealed with a filter paper containing of extracts. Electrophysiological responses from the antenna preparation to one of the six saline solutions in each test were recorded and intensity calculated by measuring the fluctuations of the signal trace on an AxoScope window panel.

As longer-living preparations can something be obtained by excising the antenna together with the head, this type of combination was also tested.

**Results**

The function of male antennae

Males with antennae completely removed did not show any WF and ZA behaviors (Fig. 4-2-1) when they were exposed to 16 FE of female extract. Eighty percent of males with half of the antennae removed demonstrated WF and ZA responses and males with intact antennae responded with 100% WF and ZA.

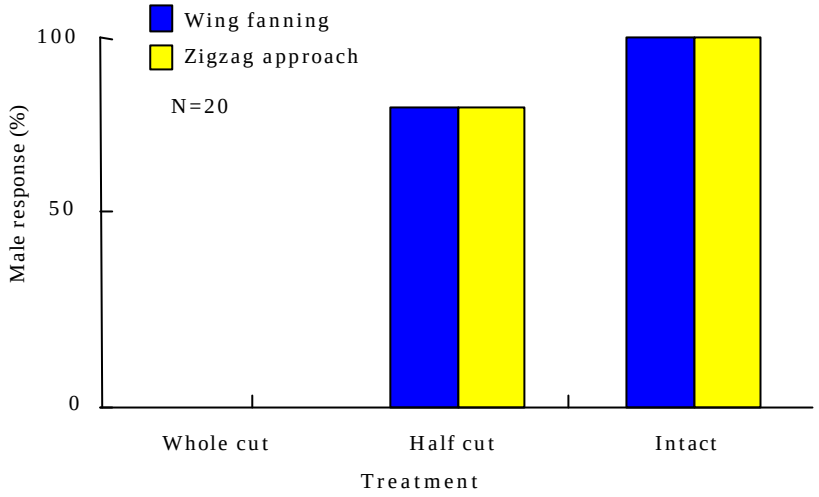



Fig. 4-2-1. Behavioral responses of males with surgically treated antennae to female extract in individual assay.

Structural differences between antenna of males and females

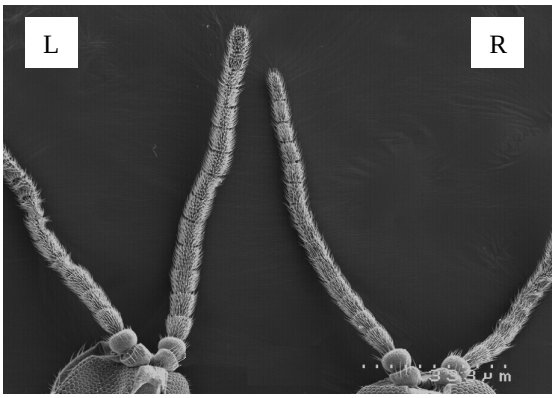
The structure of 2 male and 2 female antennae were compared under SEM (Fig. 4-2-2, a). Antennae of the male fly appeared to be longer (1000 ±58 μm) and thicker (Dia.61.4 ±2 μm) than antennae of the female (L 900 ±65 μm, Dia. 51.42±3 μm). Male antennae distinctly bore more sensilla than female antennae (Fig. 4-2-2-b, c). Ventral views of male (L) and female (R) antennae (a), 7 th flagellum of male antenna (b) and 7 th flagellum of female antenna (c).

Single antenna vs. connecting 4 antenna

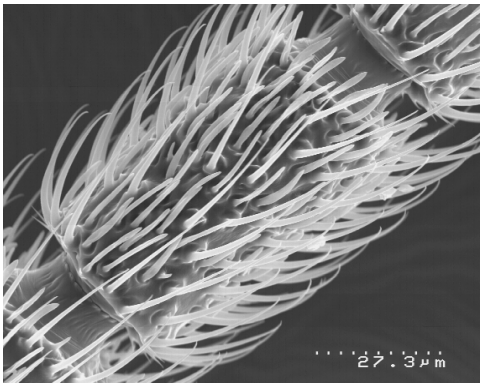
Among the 4 antenna preparation models, only model-D with a single antenna produced response signals. The recording of model 4 clearly showed difference in the electric variance between the stimuli of the crude extract and hexane control. Both responses were distinguishable from the background noise (Fig. 4-2-4).

Antenna preparations of model-A, B and C, which linked 4 antennae in a series, all failed to produce data because the antennae bridge quickly broken by the saline solution or conductive gel. The rapid evaporation of the saline was unavoidable for the preparation had to be hold under a blowing air stream while the volume of gel or saline solution was limited by space. Thus the method of linking small antenna in a series was impracticable.

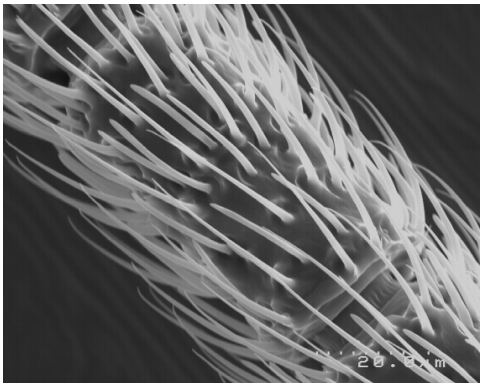
Although, responses to chemical stimuli were detected with a single antenna, randomly occurring noise during a duration of 60 min monitoring under blank running conditions caused confusion in the reading of the response (Fig. 4-2-5-b). Thus, problems still remain in the reading of EADs from this small fly.




(a)



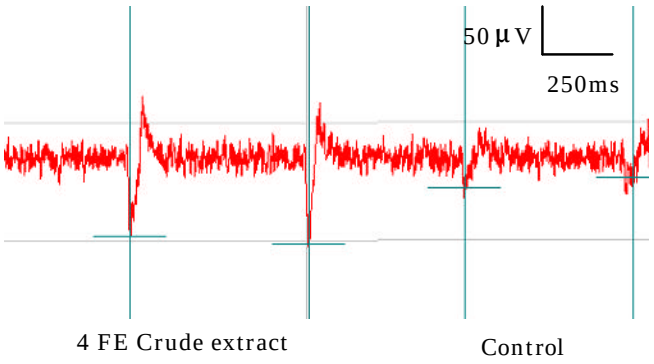

(b)



(c)

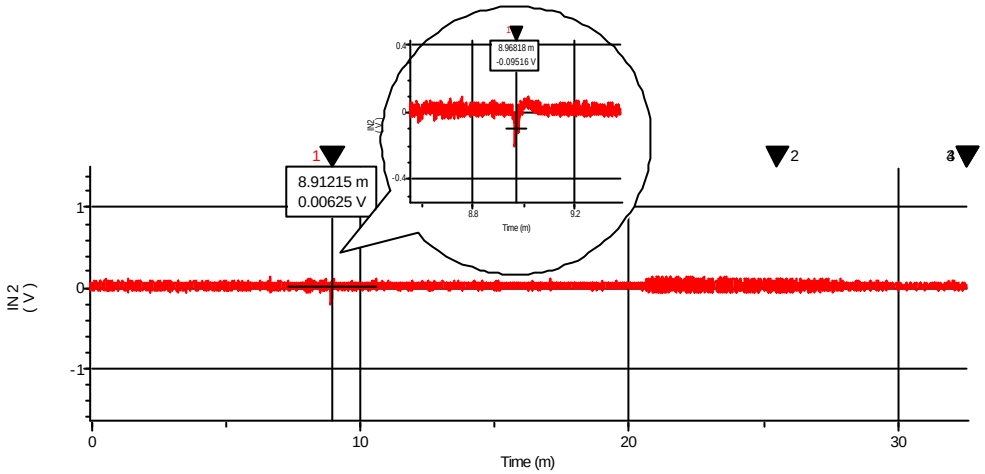
Fig. 4-2-2. Scanning electron microscopic photographs of antennae of *B. paupera*.

Fig. 4-2-3. Photograph showing a single antennal bridg between paired capillary glass electrodes.

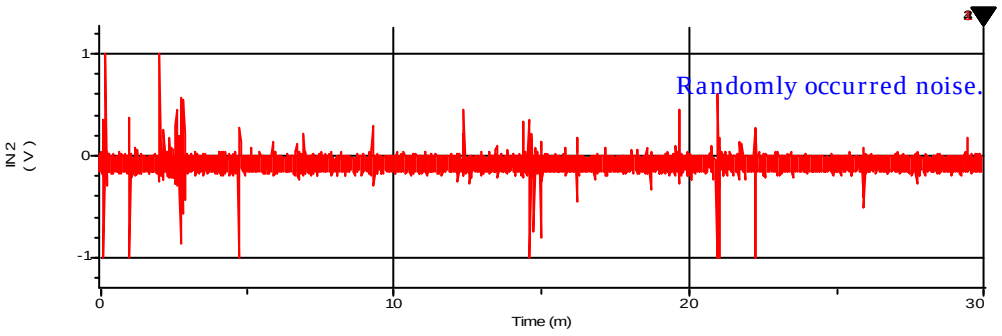



EAG

Fig. 4-2-4. EAG responses recorded from a single antenna bridged electrode. The stimuli were given in a 1 ml puff.




(a). EAD chart record of male peach moth *Conogethes punctiferalis* to E10-16:Ald.




(b). EAD chart record of male *B. paupera* to female crude extract.

Fig. 4-2-5. Comparison of continued EAG chart records of male peach moth *Conogethes punctiferalis* (a), and male sciarid fly *B. paupera* (b) under similar experimental conditions.

In order to clarify the problem, the antenna of the peach moth *Conogethes*

*punctiferalis* was tested for comparison. The *C. punctiferalis* preparation showed a stable baseline and a clear response at 8.9 min to E10-16Ald (Fig. 4-2-5-a). Therefore, this suggests the random fluctuations in noise was likely due to physiological rejective responses, e.g. lymph saline solution incompatibility to *B. paupera*.

Practicable saline composition

In repeated EAG monitoring with the previous saline solutions (Table. 4-2-1), saline 1, saline 2 and saline 6 showed activity for nearly 20 min (Fig. 4-2-6), while saline 3, saline 4 and saline 5 gave lower responses and lost activity within 5 min and 10 min. Solution 6 was higher at 5 min (22 μV) and 15 min (2.5 μV) than saline 1 (11 μV, 1.5 μV) and saline 2 (9 μV and 1 μV). But random noise occurred with saline 1 so saline 6 was better. The duration of detection was greatly improved and the antennae preparation survived 1 hr and responded to crude extract with 4.25 μV at 60 min when the antenna were used together with the head. Therefore excising the antenna together with head was considered to be adequate for EAD bioassay of *B. paupera*.

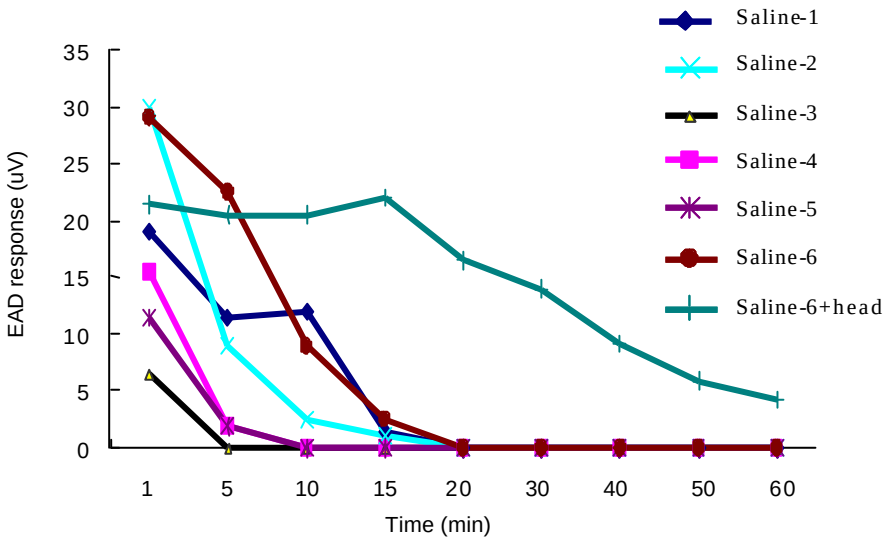



Fig. 4-2-6. Comparison of the persistence and longevity of the antennal preparation using different saline solutions.

## Discussion

Antennae of male *B. paupera* responded well to sex pheromone stimuli. Males with the antennae completely removed were unable to respond, but males with half the antennae removed were not affected. Thus loss of the ability of male antennae to respond was due to a loss of receptors and the proximal half of the

antennae was essential and sufficient for fly olfaction. The antennae of the male fly function as an important chemical receptor and are crucial for mating behavior.

The small sized antennae of *B. paupera* can be used to detect electrophysiological responses with proper setting of an electrode bridge and use of the proper saline solution. Modification of the EAD for small sized antenna (less than 1 mm) in this study appears to be the first successful measurement of sciarid flies. Nojima et al. (2003) mounted a apple maggot fly (*Rhagoletis pomonella*) head on the tip of a plastic pipet chip, formed a characteristic antennal preparation and successfully detected GC-EAD responses of the fly to host fruit volatiles. Although GC-EAD, EAG and even single receptor cell recoding techniques were established many years, it is still necessary to continue EAD recordings in various cases (Struble and Arn, 1984), especially for small sized antennae that were thought difficult to discern the responses from background noise (Roelofs, 1984). Moore (1980) suggested a technique to amplify the EAG signals based on in-series resistors, capacity theory and resulting sounds, but he merely mentioned the reform of electrode shape, selection of proper saline solution and reorganization of the amplifier capability.

This experiment failed to show practical linking of antenna in series because of the inability to prevent rapid drying up of the saline solution at the conjunction knots. The procedures used in this report, appeared to be the first time stable clear EAD recordings were obtained with such a tender small antenna (0.6 - 0.9 mm).

Comparison of the 6 composition solution, obviously showed the composition can largely determine the suitability and longevity of the antennal preparation of a certain species.

The head+antenna combination showed a distinct improvement in the duration of activity persistence. This was also an important hint for overcoming problems of the short life of small sized antennae.

Modified EAD procedures achieved sound performance and are therefore applicable for electrophysiological bioassay of sciarid flies.

## Section 3. Recording insect olfactory response by modified GC-EAD

### Materials and Methods

The instruments and methods used in this section were basically as same as that described in the previous section evaluating of saline solution excepted that a pen recorder (LR4420) was used (Fig. 4-3-1). The output antennal response signal

from the Dual-beam memory oscilloscope (VC-10, NIHONKODEN Co.) was lined into an Analogue Input Board (AIB HP) which was packed inside the GC main board panel to enable EAD signals to parallel the signals from the flame ionization detector (FID). Both FID and EAD signals were digitally recorded with a controlling computer (HP) in which was installed a HP 3365 Series Ⅱ ChemStation (ver. A. 0321J Hewlett-Packard). The GC instrument was a HP 5890 series Ⅱ plus equipped with a HP-5MS capillary column (30 m×0.25 mm, HP). Analysis was performed by holding at 50℃ for 2 min then increased temperature to 280℃ at 10℃/min and subsequent holding at 280℃ for 20 min. The injection port was at

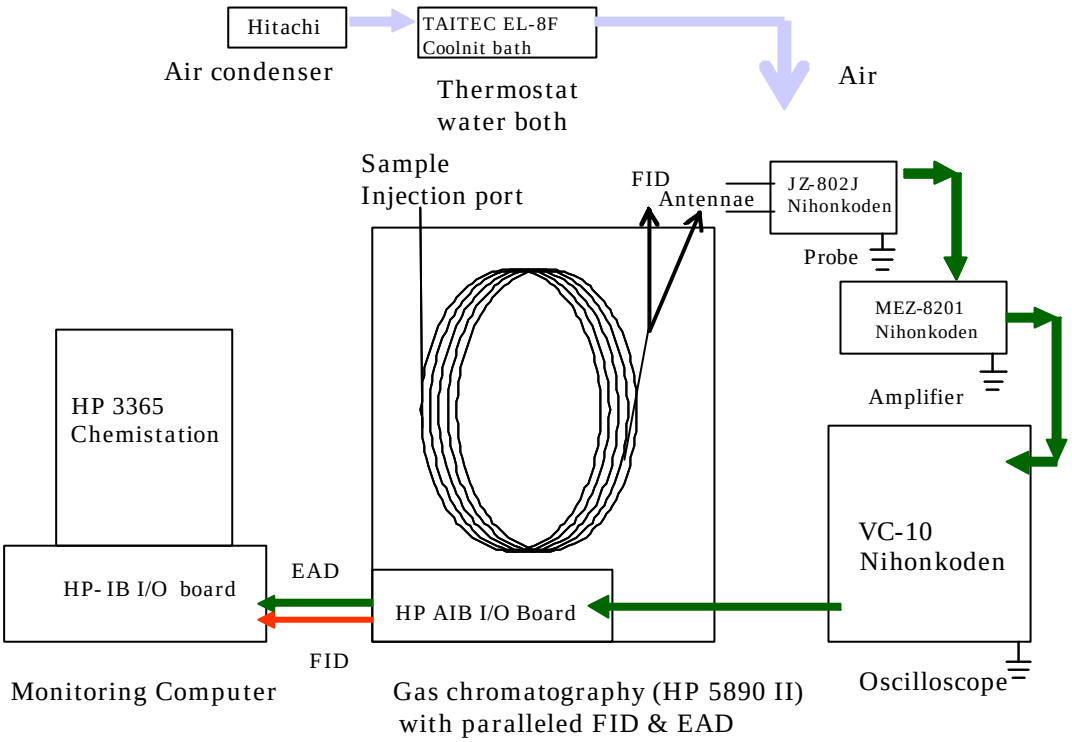



Fig. 4-3-1. Schematic of the GC- EAD system employed in the experiment.

250℃ and detection port at 300℃. The velocity of the moisturized and thermostatic (22℃) air blowing on and bringing the GC effluent towards the antenna in the conduct tube was 240 ml/min. Tracings were analyzed by integrating the FID and EAD signal chart together. Each test was started after a precheck of the antennal condition with a puff of 5FE crude extract into conduct tube at the input opening of the GC effluent.GC-EAD analysis were conducted by using antenna+head of males or females as electrode and stimulus from hexane washed crude extracts of female or male or mushroom (*L. edodes*) fruits. Each combination was repeated 3 times.

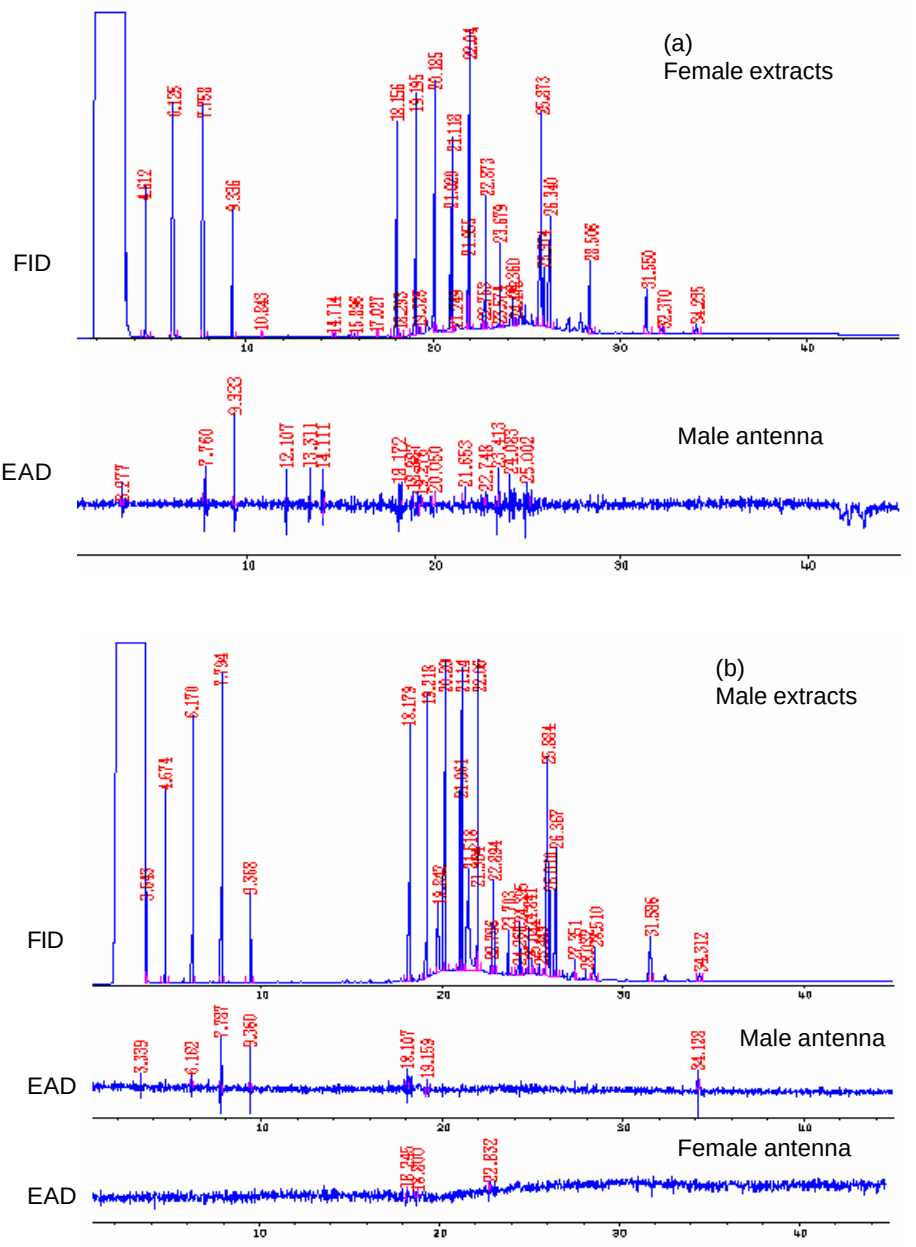



Fig. 4-3-2. GC-EAD analysis with male antenna to female extract (a), and male and female antenna to male extract (b).

**Results**

Figure 4-3-2-a shows a representative FID trace of the crude female extract and corresponding EAD response of males. Male antennae responded at about 13 points with retention times of 7.7, 9.3, 12.1, 13.3, 14.1, 18.1, 19.2, 20.0, 21.6, 22.7, 23.4, 24.0 and 25.0 min. An individual antennae preparation showed a little difference in intensity and retention times.

Male antennae showed responses at retention time 6.1, 7.7, 9.3, 18.1 and 19.1,

and 34.1 min to male extract (Fig. 4-3-2-b). Female antennae showed no recognizable response to both female and male extracts.

The representative FID traces of females and males were very similar (Fig. 4-3-2-a, b), indicating males and females share the same cuticular lipids. Similarities in EAD responses occurred at 7.7, 9.3 and 18.1 min, these 3 may be the same.

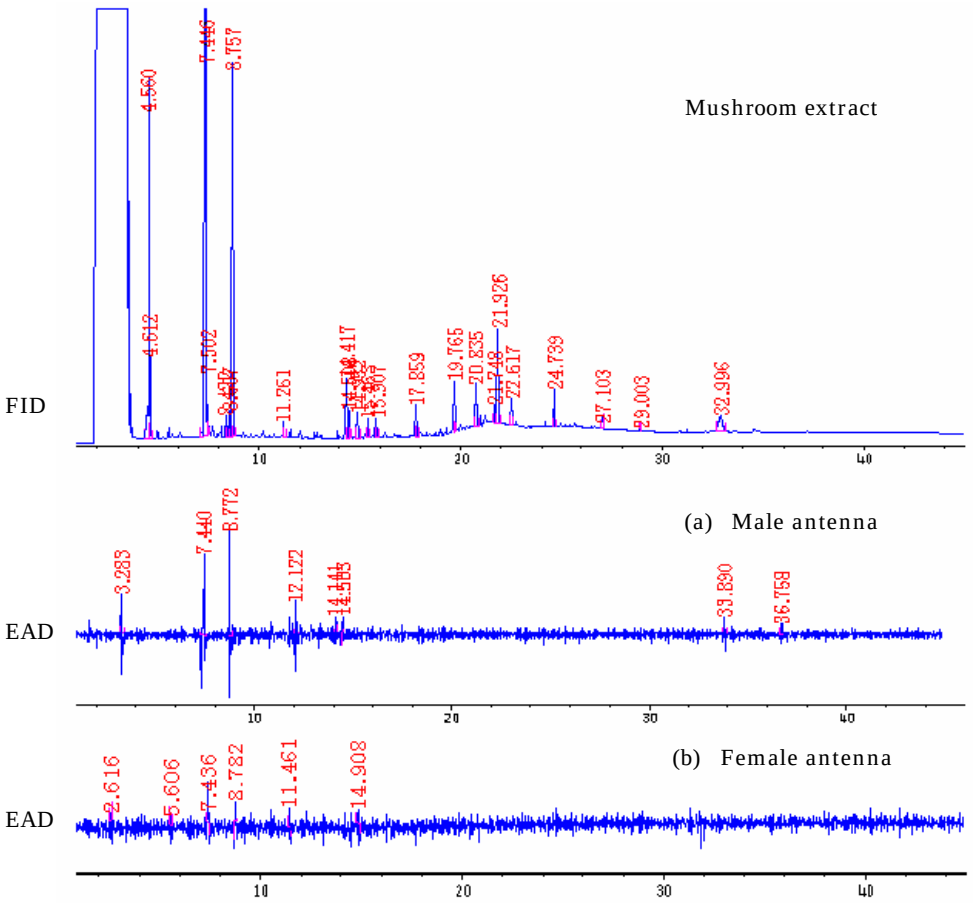



Fig.4-3-3. GC-EAD analysis of male (a), and female (b) antenna to mushroom extract.

On other hand, Fig. 4-3-3 showed both male and female antenna responded to mushroom extracts. Common responses point occurred at retention times 7.4 min and 8.7 min, but the response intensity by female antennae was obviously lower by about 1/20 of the male.

**Discussion**

The FID trace of the extracts of males and females appeared very similar in representative peaks and retention times, indicating most cuticular lipid

components were shared both sexes. Despite this assumption further identification is need as support. However, male antennae were showed distinct responses to extracts of females and males. A noticeable characteristic was that at the retention times between 10 to 18 min no large FID peaks were seen in female extracts that evoked three clear responses not detected in the male extracts. This retention time zone corresponded to Fr. 2 of the GC-PFC, which demonstrated behavioral active in male flies, described in previous section. The sensitivity and specificity of the male antenna to pheromone components provide a powerful tool to assay pheromone components and to predict structure of the pheromone components (Roelofs, 1984). The EAD responses in the 10 min to 20 min retention time zone should be high-lighted for further investigation of sex pheromone candidates.

Antennae of females not sensitive to the extracts of females or males responded to mushroom extracts. Therefore the antennae of females appear more to be sensitive and specialized for foraging or oviposition than mate finding. Antennae of female *B. paupera* not discerning response to crude extracts may be related to the physiological reasons. Howse (1998) analyzed differences in chemosensory sensitivity between *Antheraea polyphemus* male and female and listed 4 contributing factors: (a) the size and geometry of the antennae, (b) the number of pheromone-specific sensilla on the antennae, (c) the relative sensitivity of the receptor cells, (d) the convergence of chemoreceptor neurons to interneurones in the antennal glomeruli of the brain. Sensilla on antennae of female *B. paupera* were obviously smaller and fewer than on male antennae. These may explain why the antennae of male give stronger responses than antenna of females. However, the relationship of weak antenna responses to less behavioral actively in females during was still remains unclear.

EAG is not always a good predictor of behavioural responsiveness because, for example, some chemicals may stimulate neurons within the central nervous system that have inhibitory or synergistic effects and this cannot be deciphered from an EAG trace.

## Chapter Ⅴ. Identification of Sex Pheromonal Active Component

Preliminary separation and bioassay with GC-EAD showed that female sex pheromonal active components in the cuticular extracts are likely of weak polarity and very low in concentration. It was also demonstrated that females and males may share the main cuticular lipids. In order to elucidate the chemoecological function of cuticular lipids, including sex pheromones, the chemical structure of the

female sex pheromones and main cuticular lipids need to be identified.

For volatile chemical structure elucidation, mass spectrometry coupled GC-MS is undoubtedly the most sensitive. This technique provides information at three levels: molecular mass, elemental composition and structure. In certain cases the coupling of a separation step in the analysis allows information to be obtained directly on each component of a mixture.

In this chapter, the main cuticular lipids and representative GC-EAD active components were identified (Section 1., Then the bioactivity of the identified components were examined by both electrophysiological and behavioral assays (Section 2). Finally, a preliminary field test was carried out (Section 3).

## Section 1. Identification of the main cuticular lipids and EAD active components

### Materials and Methods

Gas Chromatography-Mass Spectrometery (GC-MS)

A GC-MS model (HP 5890Ⅱ) equipped with a HP-5MS column and coupled to a MSD 5972 was employed for chemical analysis and identification. The temperature program of the GC oven was same as that described for GC-EAD in section 3 of chapter Ⅳ. The MSD was performed in the EI mode (70eV, 185℃). For identification of the components corresponding to GC-PFC fraction 2, the GC-MS programmed temperature was held at 50℃ for 2 min, then increased to 130℃ at 10℃/min, then to 165℃ at 1℃/min and to 295℃ at 20℃/min followed by a 10 min-holding. The active fractions obtained from TLC (Rf 0.67), silica column chromatography (Fr. 5% e/h and Fr. 50% e/h) and GC-PFC (Fr. 1 to Fr. 3) were analyzed under the same conditions.

The identities of the chemicals were verified by acquiring commercially available or authentic standards and comparing for identical GC retention times and mass spectra. Commercially available chemicals were purchased from Wako Pure Chem. or Sigma-Aldrich, others were synthesized in the laboratory. The *n*-Aldehydes from C15 to C18 were synthesized by oxidation of the corresponding *n*-alcohols with pyridinium dichromate (PDC, Wako Pure Chem.) in $CH_2Cl_2$ (Corey and Schmidt, 1979). The *n*-Alkan-2-ons from C16 to C21 were produced by oxidizing alkan-2-ols C16 to C21 which were converted from *n*-Alkanals C15 to C20 by a Grignard reagent (methyl lithium, Kanto Chem.) with PDC. These synthetic aldehydes and ketones were purified (ca. 98%.) by subjecting them to silica gel column chromatography.

**Results**

The lipid components found in the active fractions segregated into three distinct groups according to a series of diagnostic fragment ions for each group of chemicals (Table 5-1-1). EI mass spectra of three components with early retention times in the first fraction showed $M^+$ ions at m/z 100, 114, 128, $M^+$-18, $H_2O$ ions at

Table 5-1-1. Identified of aliphatic aldehydes, hydrocarbons and 2-ketones in the cuticular lipids of the female *B. paupera*.

| *n*-Aldhyde | Retention time (min) | % | *n*-Alkane | Retention time (min) | % | 2-Ketone | Retention time (min) | % |
|---|---|---|---|---|---|---|---|---|
| C6 | 4.40 | 3.30 | C10 | 7.72 | 0.03 | C16 | 18.12 | 7.22 |
| C7 | 6.12 | 6.66 | C11 | 9.29 | 0.03 | C17 | 19.14 | 8.41 |
| C8 | 7.77 | 6.45 | C12 | 10.79 | 0.02 | C18 | 20.13 | 8.36 |
| C9 | 9.37 | 2.97 | C13 | 12.20 | 0.02 | C19 | 21.07 | 5.42 |
| C10 | 10.90 | 0.16 | C14 | 13.52 | 0.03 | C20 | 21.98 | 11.38 |
| C11 | 12.35 | 0.09 | C15 | 14.77 | 0.01 | C21 | 22.84 | 3.39 |
| C12 | 13.69 | 0.02 | C16 | 15.95 | 0.11 | | | |
| C13 | 14.95 | 0.03 | C17 | 17.09 | 0.11 | | | |
| C14 | 16.13 | 0.03 | C18 | 18.14 | 0.02 | | | |
| C15 | 17.26 | 0.06 | C19 | 19.16 | 0.03 | | | |
| C16 | 18.35 | 0.18 | C20 | 20.14 | 0.05 | | | |
| C17 | 19.37 | 0.16 | C21 | 21.06 | 0.07 | | | |
| C18 | 20.36 | 0.07 | C22 | 21.95 | 0.04 | | | |
| | | | C23 | 22.80 | 0.17 | | | |
| | | | C24 | 23.63 | 0.08 | | | |
| | | | C25 | 24.41 | 0.10 | | | |
| | | | C25 | 25.18 | 0.13 | | | |
| Total | | 20.02 | | | 1.03 | | | 44.2 |

(Percentage of each component was assessed by reference to the corresponding GC peak area in the crude extracts of 80 FE)

m/z 82, 96, 110 and a hydrocarbon pattern suggesting *n*-hexanal, *n*-heptanal and *n*-octanal. These were followed by a series of aliphatic homologues with chain lengths of 9 to 18 carbons with $M^+$ or $M^+$-18, $M^+$-28 (CO), $M^+$-44 ($CH_2CHOH$) ions originating from the corresponding molecules from C9 to C18. Mass spectra and retention times coincided well with those of authentic saturated aliphatic aldehydes

with chain lengths of 6 to 18 carbons. The *n*-Alipphatic aldehydes of C6 to C18 comprised 20.2% of the total lipids of females body surface and ca.65% of the total aldehydes consisted of *n*-hexanal to *n*-nonanal.

The most abundant components in the active fraction showed $M^+$ ions at m/z 240, 254 and 268. They were also characterized by m/z 225, 239, 253 for $M^+$-15 ($CH_3$) ions and 197, 211, 225 for $M^+$-43 ($RCO^+$) ions. A fragment ion at m/z 58 by

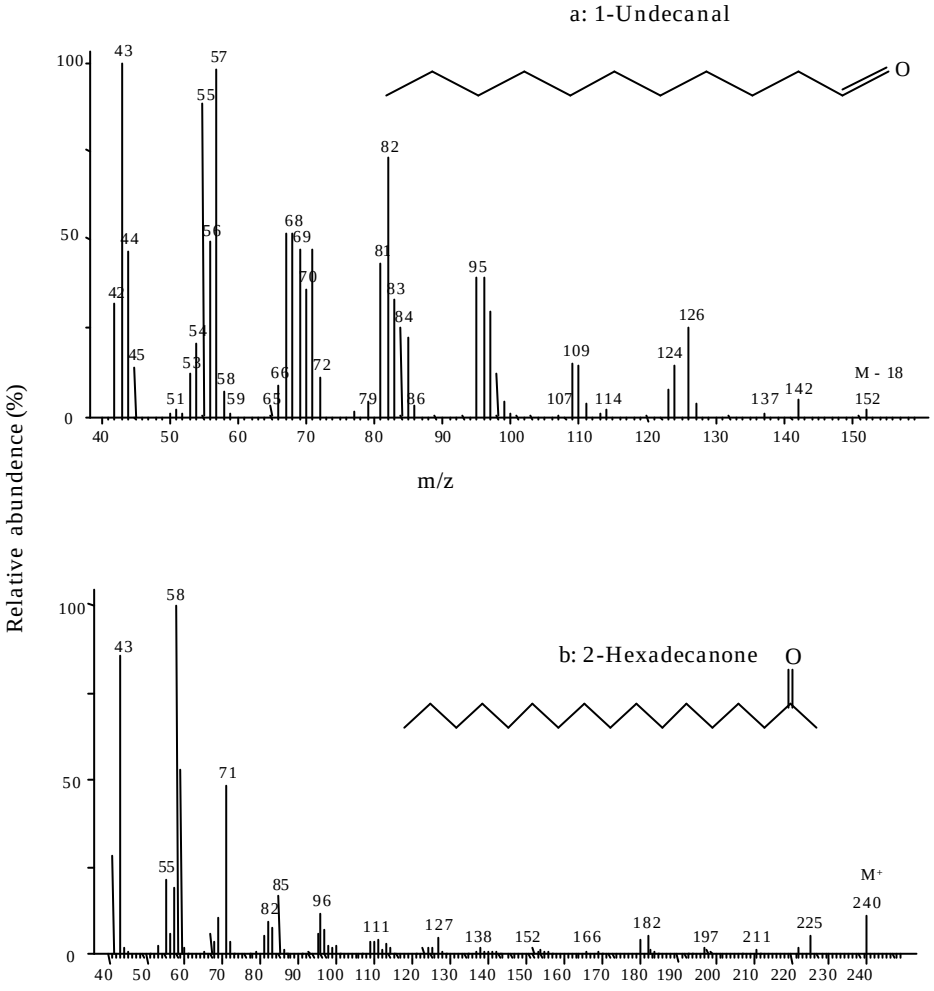



Fig. 5-1-1. Mass spectrum of 1-undecanal (a), and 2-hexadecanone (b) in the female crude extract.

McLafferty rearrangement was also commonly observed in the mass spectra. These mass spectra data allowed for the elucidation of 2-aliphatic ketones with $C_nH_{2n}O$ structure. Their long chain homologues were also identified as major components of other fractions. These ketones comprised 44.2% of the total lipids of the females body surface.Figure 5-1-1 presents mass spectrum of (a) 1-undecanal and (b) 2-hexadecaone. Verbalization of the mass spectrum of other identified chemicals listed in Table 5-1-1 are omitted due to the similarities between the homologous.

The spectrum of *n*-hydrocarbons are also omitted sine they are acquaintance to common.

EI mass spectra for other components of the active fractions showed a typical fragmentation pattern characterized by peak clusters consisting of $C_nH_{2n}$, $C_nH_{2n+1}$, $C_nH_{2n-1}$ and the corresponding peaks of each cluster were 14 ($CH_2$) mass unites apart. Their molecular ions were at m/z 226 (C16), 240 (C17), 254 (C18), 268 (C19), 282 (C20), 296 (C21), 310 (C22), 324 (C23), 338 (C24), 352 (C25) and 366 (C26), respectively. Corresponding authentic *n*-alkans were identical with these components in the mass spectra as well as retention times on GC. The total *n*-hydrocarbons identified was only 1% of the total lipids of females body surface.

The components were labeled in a total ion chromatography of the female extract with the corresponding GC-EAD trace in Fig. 5-1-2. The retention times 7.7 min, 9.3 min, 18.1 min and 19.2 min corresponded to octanal, nanonal, 2-haxadecanone and 2-heptadecnone, respectively. Aldehyde C6-C9 and 2-ketone

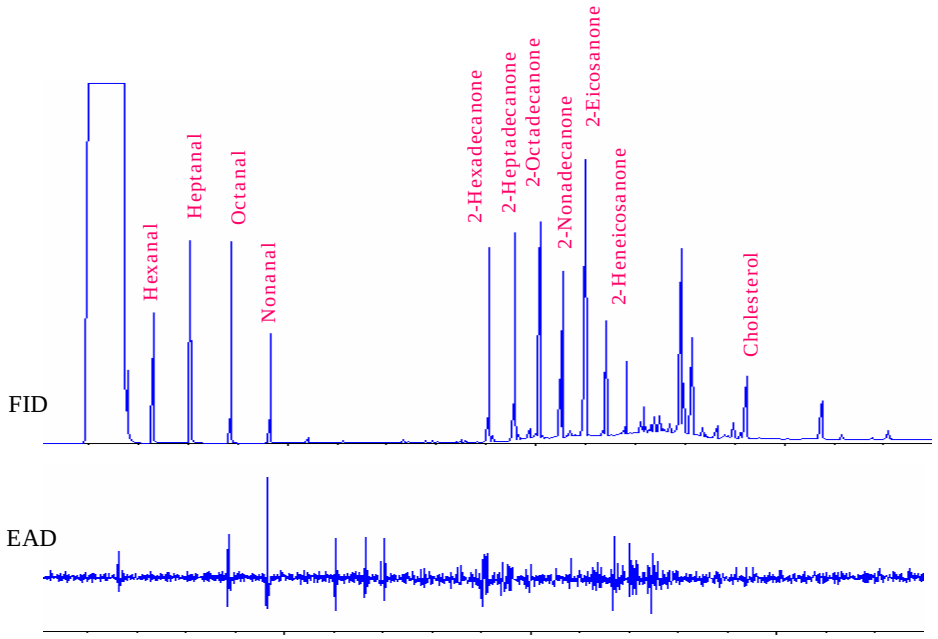



Fig. 5-1-2. High profile components identified in female cuticular lipids and their GC-EAD activity.

C16-C21 were identified in both female and male cuticular extracts. Cholesterol was also identified from the female extract by the same method.Three EAD responses at retention times 12.1 min, 13.3 min and 14.1 min corresponding to Fr. 2 of the GC-PFC fraction are still being identifed (Fig. 5-1-3). In a preliminary database search (The National Institute of Standards and Technology NIST, US) by the mass spectral search program for the NIST/EPA/NIH Mass spectral library,

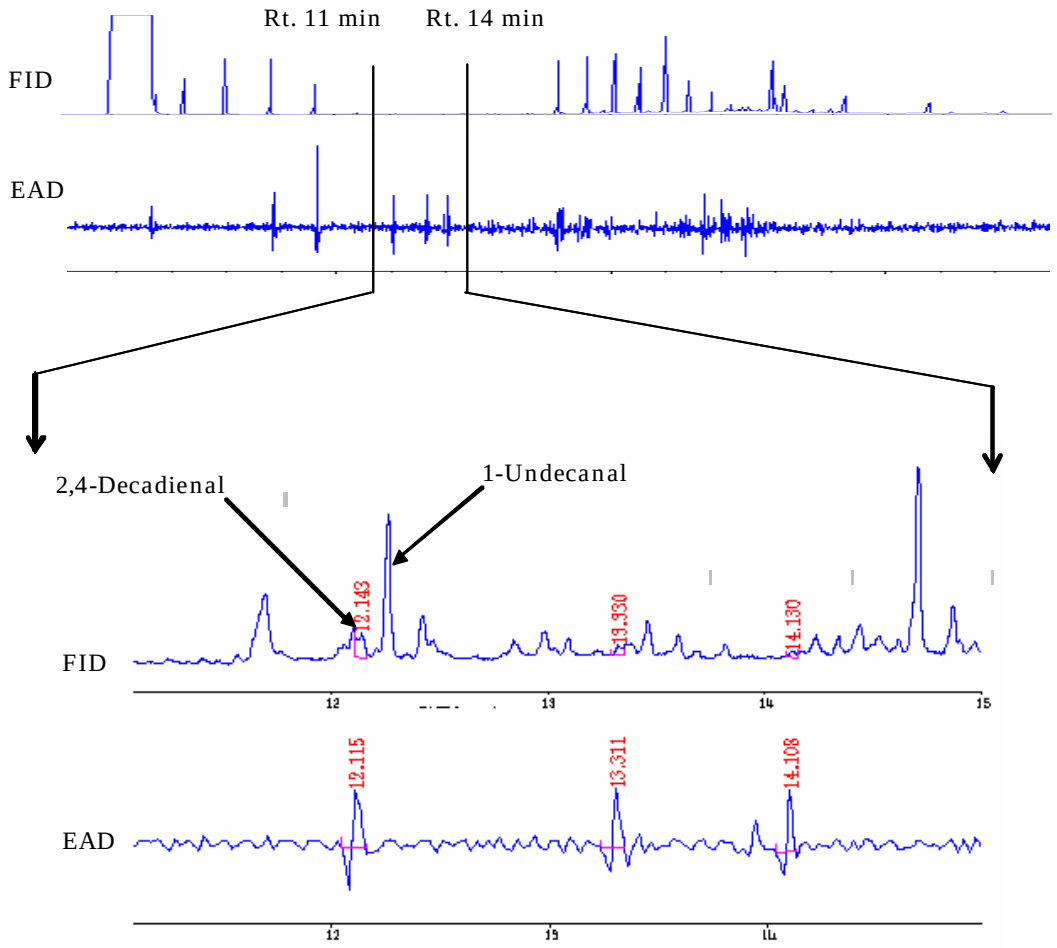
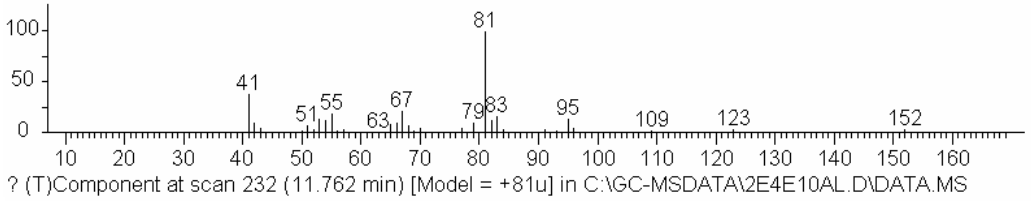



Fig. 5-1-3. Retention time 11-14 min of GC-EAD trace of male antenna to the crude female extracts.

(a)




(b)

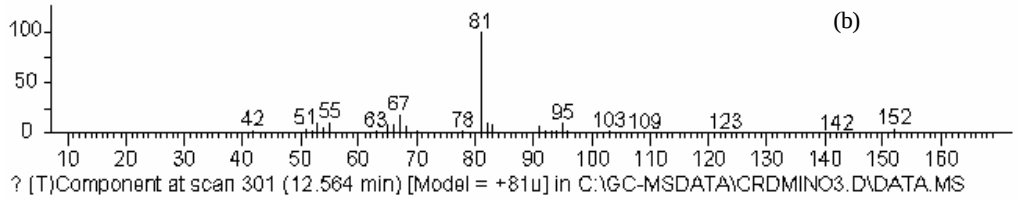



Fig. 5-1-4. Mass spectra of 2*E*,4*E*-decadienal (a), and a GC-EAD active component (b) in the female crude extracts.

version 1.7 and automated mass spectral deconvolution and identification system (AMDIS 2.0 by NIST), 2*E*, 4*E*-docadiencal matched the mass spectra of the component corresponding to the EAD response at retention time 12.1 min. Subsequently, the mass spectra of authentic 2*E*, 4*E*-docadiencal was compared (Fig. 5-1-4). However, the examination of a commercially available 2*E*, 4*E*-docadiencal of

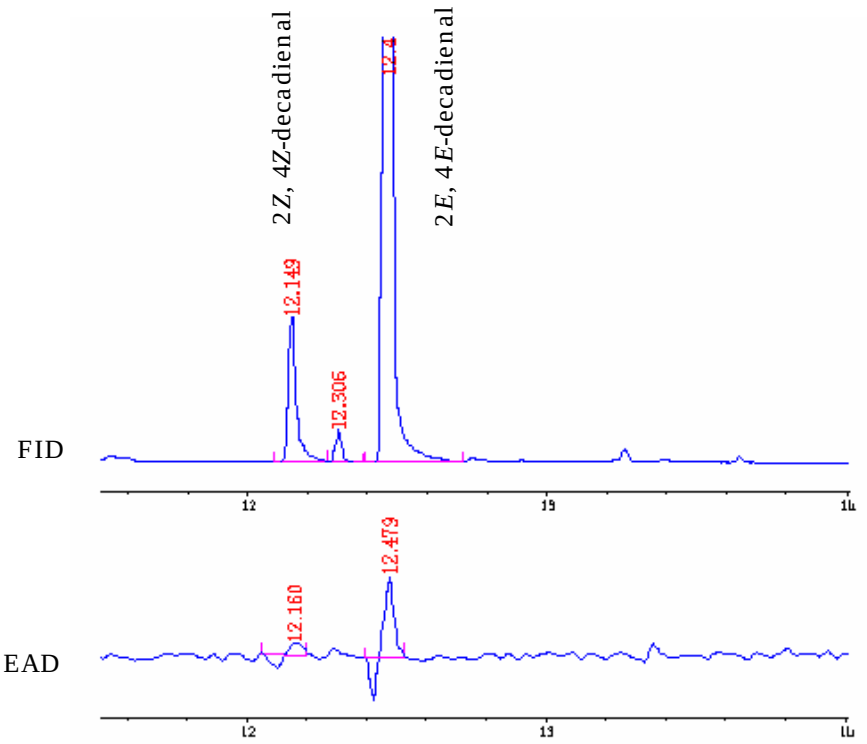



Fig. 5-1-5. GC-EAD responses of male antenna to authentic 2*E*,4*E*-decadienal and 2*Z*, 4*Z*-decadienal.

90% purity (Aldrich) by the individual assay showed no behavioral responses in males (Table 5-2-2). Further investigation of antennal activity of the synthetic chemical by GC-EAD, suggested the retention time 12.1 min was concord to 2*Z*, 4*Z*-decadienal not 2*E*, 4*E*-decadienal, despite both caused male antennal responses (Fig. 5-1-5). It is then speculated the EAD active component at retention time 12.1 min is 2*Z*, 4*Z*-decadienal. However, the mass spectrum of the substances corresponding to 13.3 min and 14.1 min remained unclear due very low in ions abundance.

**Discussion**

Rarely has a series of regular homologue chemicals been reported from one species, thus the cuticular substances identified in *B. paupera* appeared to be characteristic. However, this may just be due to others species not yet being analyzed at the same level. Kostelc et al. (1979) reported a series of standard hydrocarbons C15 - C26 evoked sex pheromonal activity in *L. mali* males and the heptadecane was the major attractant active at 1 μg to 1 ng. However, the behavioral indicators, e g. WF, ZA and LO, were not provided for comparative discussion.

The cuticular hydrocarbons (HC) were composed primarily of *n*-alkanes, alkenes, and methyl-branched components. They are major components of insect epicuticular lipids and are distributed throughout the epicuticular surface of all insects (Howard, 1993). The primary function of cuticular HC is to protect insects from water loss, and have been recognized to be important regulators of cuticular permeability. But they also serve as communication signals directly as sex pheromone. For example, (*Z*)-9-tricosene serves as a sex pheromone in the house fly *Musca domestica* (Carlson et al., 1971) and (7*Z*, 11*Z*)-7,11-heptacosadiene for *Drosophila virilis* (Oguma et al., 1992).

Aldehyde components have merely appeared as lists of semiochemicals in Diptera. Only one aldehyde, hexanal, has been shown to be eluted by adult male as sex pheromone to attract the female in the flesh fly *Sarcophaga bullata* (Parker) (Gerard et al., 1979). Hexanal was found in the cuticular extract of female *B. paupera* and their male fly along with heptanal, octanal and nonanal.

Ketones have been shown as aggregation pheromones in a number of dipteran species. (*Z*)-10-heptadecen-2-one is as aggregation pheromone in *Drosophila martensis, D. buzzatii* and *D. serido* and 2-pentadecanone an aggregation pheromone in *D. busckii*, and *D. hydeei* (Moats et al., 1987; Bartelt et al., 1985), *Drosophila hydei* (Moats et al., 1987), *D. martensis* (Schaner and Jackson, 1992) and *D. serido* (Schaner and Jackson, 1992). Ketone substances of C16-C21 were found as most abundant contents in the cuticular extracts of both sexes of *B. paupera*..

Three groups of cuticular components together making about 65% of the contents were identified and about 35% remain unidentified. According to the retention times of the components, the ketones did not show behavioral activity in any preliminary analysis. Therefore , their identification was postponed.

As shown in Fig. 5-1-3, the most EAD active components also remain uninvestigated because of very low amounts. Their identification is thought

significant and thus need to be continued.

## Section 2. Behavioral and electrophysiological activity of identified components

### Materials and Methods

Behavioral activity of identified components

The group assay was employed to examine the behavioral activity of the identified components. In order to shorten the time interval between tests, a mini fan was attached beneath the bottom of the cage to vent the air.

Responses of males to each single identified component which listed in Table 5-1-1, their mixtures furthermore and some structurally similar commercial chemicals (see Table 5-2-1) were tested.

1-Udecanal was tested at range of dosages from 0.1 pg to 10 μg. The amount of 1-undecanal per female was estimated by comparison of the peak area of 1-undecanal in gas chromatography (GC) of the crude extracts with the peak area of a series GC injections with different concentration of 1-undecanal.

Electrophysiological activity of identified components

Identified components were analyzed for GC-EAD activity to male antenna by injecting 3 mixtures of *n*-hydrocarbons C10-C26, 1-aldehydes C7-C18 and 2-ketone C16-C21 separately following the procedures described in section 3 of chapter Ⅳ. The intensity of the responses of individual components contained in the mixtures were determined measuring by the voltage with a HP 3365 computerized chemstation.

$LiAlH_4$ reduction of crude female extract and aldehydes: Crude female extract (2000 FE) was added to a solution of 4 ml dry ethyl ether and 0.1 g $LiAlH_4$, the solution was refluxed with magnetic stirring for 2 h at 0℃ and 4 h at room temperature. Then 2-3 drops of 5 M HCl and 2 ml distilled water were added to the reactant to deactivate remaining $LiAlH_4$. The reactant was obtained by filtering the up ethyl ether layer and concentrated to a proper volume under a $N_2$ stream. Ten mg of the aldehyde (C7-C18 of equal) mixture was treated with $LiAlH_4$ by the same procedures. The behavioral activity of both samples was checked by the group assay and antennal level activity by GC-EAD analysis as described in section 3 of chapter Ⅳ.

**Results**

Activity of individual authentic chemical

Figure 5-2-1 shows the behavioral pheromonal activity of each compound in 3 vertically arranged bar-graphs of *n*-aldehydes (a), *n*-hydrocarbons (b) and 2-ketones (c). Among the 12 aldehyde homologues, male WF, ZA and LO responses were observed to *n*-decanal (LO, 10.6%), *n*-undecanal (LO, 32%), *n*-dodecanal (LO, 24%) and *n*-tridecanal (LO, 14.2%). The maximal responses were observed to *n*-undecanal. In *n*-hydrocarbon homologues of C10 to C26, only undecane (LO, 2%), dodecane (LO, 22%) and tridecane (LO, 2%) evoked responses in males. Dodecane was comparatively more effect than the other compounds. Six 2-ketone homologues (C16-C21) that were dominant components of the crude extracts showed no pheromonal activity.

Responses of males to a range of 0.1 pg to 10 μg of *n*-undecanal in the group assay are shown in Fig. 5-2-2. Maximal responses of 67.5% WF and 60% ZA were observed at a dose of 1 μg, but a LO response of 5%. The maximal LO response was

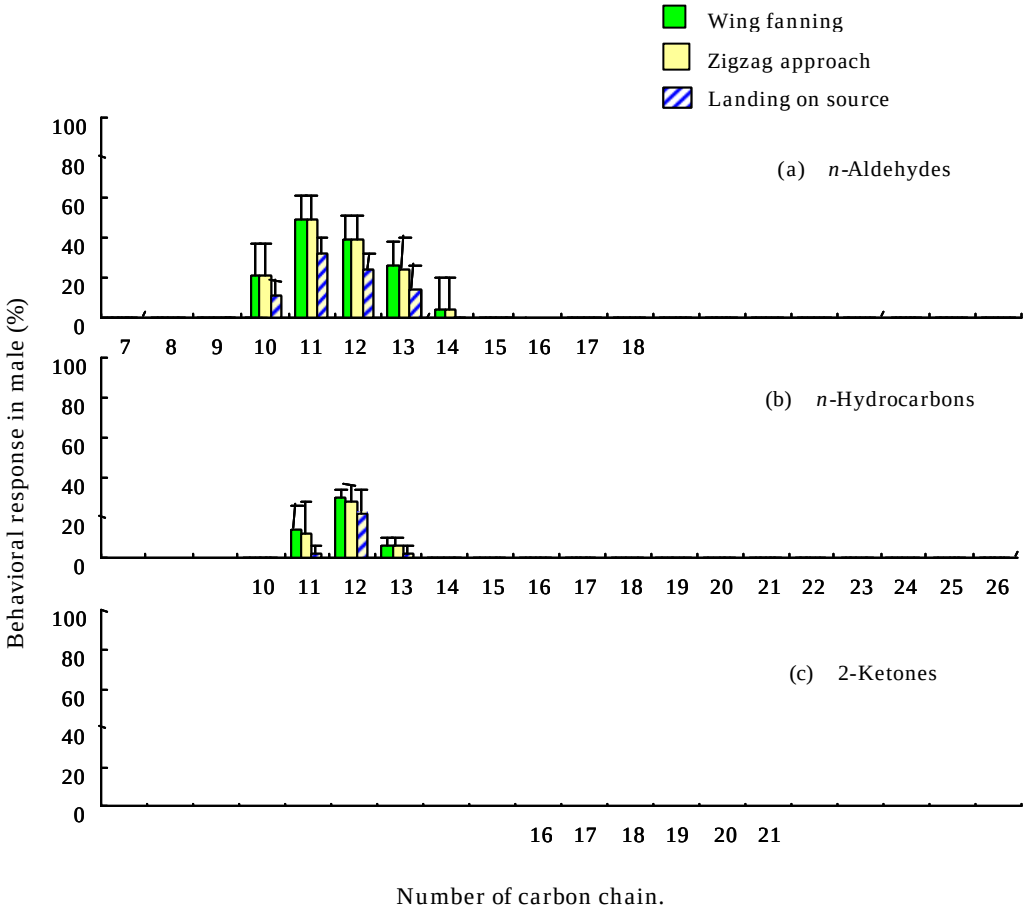



Fig. 5-2-1 Behavioral responses of male flies to *n*-aldehydes (a), *n*-hydrocarbons (b), and 2-ketones (c).

however, at 100 ng (25%). These behavioral responses by the male flies decreased at higher doses and a threshold was observed at 1 pg.

The amount of 1-undecanal per female was estimated to be about 2 pg.

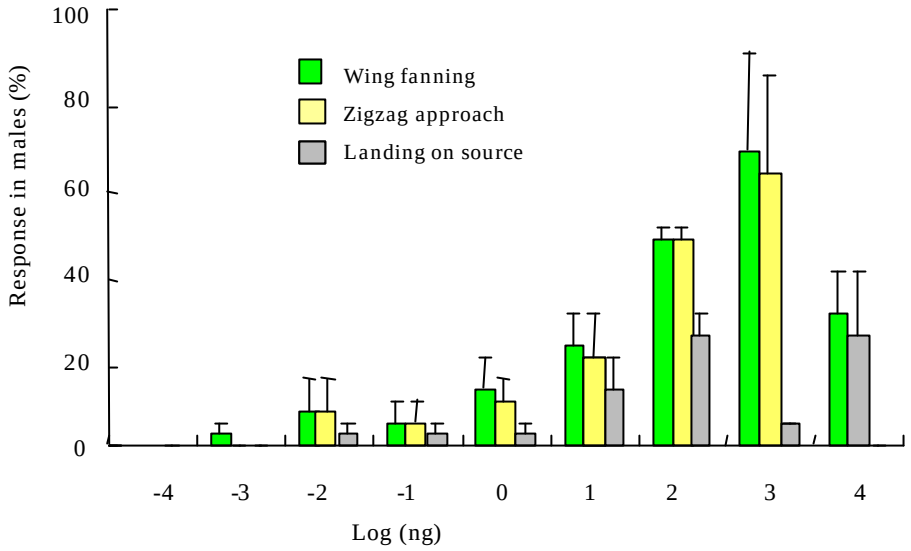



Fig. 5-2-2. Response of *B. paupera* males to different amounts of 1-undecanal in the group assay.

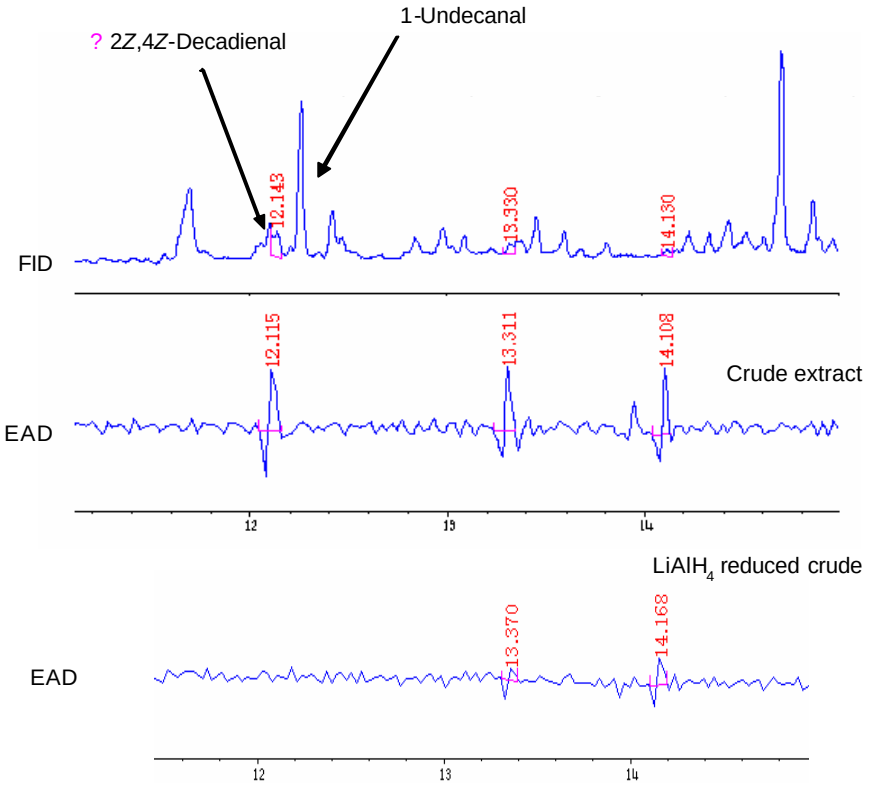



Fig. 5-2-3 Comparison of GC-EAD responses of male to crude female extract before and after LiAlH4 reduction.

Mixtures of authentic chemicals (Table 5-2-1), a mixture of *n*-aldehyde C7-C18 (equal ratio) and *n*-aldehyde C10-C13 (natural ratio), elicited comparatively higher responses (LO, 26.7% and LO, 26.5%). Similar responses were observed with a mixture of *n*-aldehyde C11,C12 and *n*-hydrocarbon C11,C12 (LO, 25) at a natural ratio. The mixture of *n*-alkanes from C10 to C26 and the mixture including *n*-alkene C11-C14 elicited clear male fly responses of LO, 15.3% and 18.3%. Three commercial undecenals also showed LO behavioral activity of 20%, 13.7% and 15%. On the other hand, the crude extract (15 FE) showed 76.7% WF, 75% ZA and 58.3% LO, whereas the hexane control, 2-ketone mixture and alcoh-1-ol (C11-C14) mixture didn't elicit any response in males. This showed the synthetics just elicited as much as a 50% response rate of the crude extracts.

Table 5-2-1. Sex pheromonal activity of synthetic in a group bioassay.

| Test Sample | Ratio & Amount | Response in males (mean ± SD%) | | |
|---|---|---|---|---|
| | | Wing fanning | Zigzag approach | Landing on source |
| Crude | 15 FE | 76.7 ±0.6 | 75.0 ±1.0 | 58.3 ±0.6 d |
| Hexane (Control) | 100 ng | 0 | 0 | 0 a |
| 1-Ald (C:7 - C:18) | Equal, 50 ng | 51.7 ±0.6 | 51.7 ± 0.6 | 26.7 ±1.5 c |
| *n*-HC (C:10 - C:26) | Equal, 50 ng | 23.3 ±0.6 | 21.7 ±0.6 | 15.3 ±1.6 bc |
| 2-Ketone (C:16 - C:21) | Equal, 50 ng | 0 | 0 | 0 a |
| 1-Ald (C: 10 - C:13) | Natural, 150 ng | 50.0 ±2.6 | 48.3 ±2.1 | 26.5 ±4.0 c |
| 1-Ald (11,12): HC (11,12) | Natural, 150 ng | 46.7 ±0.6 | 46.7 ±0.6 | 25.0 ±1.0 c |
| 1-Alkene (C:11 - C:14) * | Equal 50, ng | 28.3 ±1.5 | 26.7 ±1.5 | 18.3 ±0.6 bc |
| Alcoh-1-ol (C:11 - C:15) * | Equal 50, ng | 0 | 0 | 0 a |
| Cis-8-Undecenal * | 100 ng | 35.0 ±2.0 | 35.0 ±2.0 | 20 ±1.0 bc |
| trans-2-Undecenal * | 100 ng | 23.3 ±0.6 | 23.3 ±0.6 | 13.7 ±1.5 b |
| 10-Undecenal * | 100 ng | 18.3 ±1.5 | 18.3 ±1.5 | 15.0 ±1.0 b |
| 2*E*, 4*E*-Decadienal | 100 ng | 0 | 0 | 0 a |

* Starred samples were chemicals not found in female cuticular lipids.
Different letters indicate significant differences in responses tested by chi-square analysis at $P < 0.05$.
(n=20 3 replicates)

Antennal olfactory responses to identified components

Responses of the male antennal receptors to three groups of authentic homologues, 1-aldehydes (C7-C18), *n*-hydrocarbons (C10-C26) and 2-ketone

(C16-C21), is shown in Table 5-2-2. The *n*-Aldehyde homologues from *n*-heptanal to *n*-octadecanal elicited almost the same level of antennal electrophysiological responses in the male

Table 5-2-2. Electrophysiological responses by antenna of male to female cuticular lipid components.

| *n*-Aldehyde | EAG (μV ± SD) | *n*-Alkane | EAG (μV ± SD) | 2-Ketone | EAG (μV ± SD) |
|---|---|---|---|---|---|
| 6 | 5.6 ± 3.4 | 10 | 15.5 ± 1.2 | 16 | 10.3 ± 2.4 |
| 7 | 22.8 ± 11.2 | 11 | 21.2 ± 3.6 | 17 | 5.2 ± 0.8 |
| 8 | 36.6 ± 15.8 | 12 | 16.5 ± 1.5 | 18 | 7.6 ± 1.8 |
| 9 | 33.0 ± 10.1 | 13 | 10.1 ± 0.5 | 19 | 8.8 ± 2.1 |
| 10 | 31.9 ± 7.1 | 14 | 10.9 ± 3.6 | 20 | 10.8 ± 2.6 |
| 11 | 39.9 ± 11.4 | 15 | 6.8 ± 1.2 | 21 | 7.3 ± 1.8 |
| 12 | 31.4 ± 4.6 | 16 | 7.0 ± 2.1 | | |
| 13 | 27.2 ± 5.4 | 17 | 4.4 ± 1.0 | | |
| 14 | 43.7 ± 16.1 | 18 | 4.2 ± 0.2 | | |
| 15 | 42.6 ± 13.4 | 19 | 2.1 ± 1.6 | | |
| 16 | 18.7 ± 5.7 | 20 | 1.4 ± 1.6 | | |
| 17 | 30.3 ± 8.5 | 21 | 3.1 ± 2.1 | | |
| 18 | 19.0 ± 4.3 | 22 | 5.0 ± 1.2 | | |
| | | 23 | 5.0 ± 1.6 | | |
| | | 24 | 2.9 ± 1.1 | | |
| | | 25 | 1.6 ± 1.1 | | |
| Total | 31.4 ± 8.4 | Total | 7.3 ± 4.8 | Total | 8.3 ± 2.1 |

Table 5-2-3. Comparison of pheromonal activity of crude extract and aldehyde mixture before and after LiAlH4 reduction.

| Treatment | | Response of males (mean ± SD%) | | |
|---|---|---|---|---|
| | | Wing fanning | Curling abdomen | Landing on source |
| Crude extract | Before (15 FE) | 76.7 ± 0.6 | 75.0 ± 1.0 | 58.3 ± 0.6 |
| | After (30 FE) | 50 ± 1.5 | 50 ± 1.5 | 30 ± 1.0 |
| *n*-Ald: C7-18 | Before (each 50 ng) | 51.7 ± 0.6 | 51.7 ± 0.6 | 26.7 ± 1.5 |
| | After (each 50 ng) | 0 | 0 | 0 |



(n=20 3 replicates)

flies and averaged 31.4 μV. In the hydrocarbon homologues (C10 to C26), the responses showed a high response around C10-C14 with a maximal at C11 of 21.2 μV, then showed a decrease response sensitivity to an average 7.3 μV for C14-C26. Male antennae responded to 2-ketones at a lower level with an average 8.3 μV. These GC-EAD responses showed a similar pattern to that behavior responses such as hydrocarbons C11-C13 were effective and 2-ketones showed no effect. The antennae appear to respond more sensitively to the aldehyde structure than other functional groups.

Pheromonal activity of $LiAlH_4$ reduced female crude extract and aldehyde mixture

After the crude female extract was reduced with $LiAlH_4$, the activity of WF, ZA and LO decreased to 50%, 50% and 30%, respectively (Table 5-2-3). About a 25% decrease from before reduction. On the other hand, the aldehyde mixture completely lost activity in male flies.

Monitoring the reactants by GC-EAD showed 2 EAD active points at 13.3 min and 14.1 min remained active (Fig. 5-2-3), thus this 2 EAD response components are highly considered to the key factors.

**Discussion**

The *n*-Hydrocarbons arranged from C11-C13 were observed to be behaviorally active in male *B. paupera* , however, these components only evoked weak responses even lower than undecanal. Heptadecane as a sex pheromone for *L. mali* (Kostelc et al., 1979) was questioned by Gotoh et al. (1999, but the existence of a series of standard hydrocarbons, C15-C26, is thought common to *B. paupera.* The n-hydrocarbons that showed behavioral activity in males was not considered to be the main sex pheromone of *B. paupera* because both behavioral and electrophysiological activity was lower than to aldehydes.

GC-EAD responded to 2-hexadecanone and 2-heptadecanone, but the male antennae seemed not to be tuned specially to ketones. The 2-ketones evoked obviously lower responses than the aldehydes and hydrocarbons when the same amount were checked for antennal activity using authentic chemicals. However, whether these ketones function as aggregation cues remains unclear, but in all behavioral bioassay males showed no chemotaxis to 2-ketones.

Aldehyde components have merely appeared as a list of semiochemicals identified in Diptera or Lepidoptera. In only one example, the males flesh fly *Sarcophaga bullata* (Parker) elute hexanal as a sex pheromone attractive to females (Gerard et al., 1979). Hexanal was also found in extracts of female *B. paupera*, along

with heptanal, octanal and nonanal and were also shared by male flies. Despite octanal and nonanal consistently evoking stable electrophysiological responses in male antennae no behaviorally active was observed. The *n*-aldehydes C6-C18 were identified to be exist in female cuticular substances, but only C10-C13 aldehydes elicited ambiguous sex pheromonal behavior in males. Minor components of C10-C18 were not found in male extracts. Further experiments are still needed to elucidate the significance of this series of chemicals existing in female *B. paupera* extracts and their ability to elicit behavioral response in males at the above specific amounts.

Aldehyde components generated comparatively stronger antennal responses than hydrocarbons and 2-ketones and the aldehydes with carbon chain lengths of 10 to 13 were more sensitive than longer or shorter hydrocarbons, suggesting the chemoreceptors on the antennae respond to the aldehyde functional group and structure with carbon chain lengths near C11. Roelofs and Carde (1974) used a series of clues to successfully deduce the structure of the sexual pheromone of the lesser apple worm moth, *Grapholitha prunivora*, based on the highest EAG response given to 12-carbon atom compounds, acetate functional groups and Z isomers. The EAD responses of antennae of male *B. paupera* showed that female cuticular 1-undecanal expressed maximal bioactivity of the aldehydes and dodecane expressed maximal bioactivity of the hydrocarbons. Non female origin 1-undecene and 3 undecenals were also behaviorally active. On the other hand these structurally similar chemicals elicited male behavioral responses at a low rate may indicated they are not the main components of the female sex pheromone. Furthermore, 1-undecanal was demonstrated to be the most effective component among all identified cuticular lipids to stimulate male behavioral responses. The threshold dose needed in the individual assay was 10 ng. However, 2 pg 1-undecanal per female is below the response threshold. Therefore, 1-undecanal alone is not considered the main sex pheromone factor.

After reduction by $LiAlH_4$, crude female extracts remained 25% activity to elicit WF and ZA of 50% and LO of 30% in males. Meanwhile the $LiAlH_4$ reduced aldehyde mixture completely lost activity. These results contradict results of the electrophysiological bioassays suggesting the antennae are likely sensitive to substances possessing carbon chain lengths of about C11-C12 and aldehyde structures. However, it is reasonable to think that the unidentified sex pheromone component would be a hydrocarbon or terpene with a carbon chain length near to C11, because the hydrocarbons C10-C13 also elicited strong antennal responses. However, the results of the diagnostic reaction indicate that except for the carboxyl

structured compounds including aldehydes, 2-ketones and carboxylic acids, there exist other chemical(s) that are important as sex pheromone components. Further studies are needed to clarify the components of the female sex pheromone. The EAD active components at retention times 13.3 min and 14.1 min remained active even after $LiAlH_4$ reduction, thus these 2 components are highlighted as candidates for final elucidation of the sex pheromone of *B. paupera*.

Homologous aldehydes and hydrocarbons which not from female origin but elicited behavioral responses are possible due to being structurally similar to the sex pheromone components. Elucidation of remained GC-EAD active component is apparently essential for explain the biological significance of identified lipids.

## Section 3. Preliminary field trapping test

### Materials and Methods

Field trap experiments were conducted at a mushroom (*Agarisuc bisporus*) house at Unakami in Chiba Prefecture, Japan.

Sticky traps (24 cm x 30 cm, Takeda Chem. Japan) were hung 50 cm above compost beds in a mushroom house (14 m×7 m, 17°C) (Fig. 5-3-1). Three types of lures (See Table 5-3-1), aldehyde made from *n*-undecanal (1 mg), aldehyde mixture C7-C18 in equal ratios to a total of 10 mg and aldehyde mixture C10-C13 in natural ratio (53.7: 31.3: 6.4: 8.5) to a total of 10 mg, were used. Test chemicals *n*-undecanal, Ald: C10-C13, Ald: C7-C18 and Hexane were dissolved in hexane and loaded onto a rubber septa (No. 4, Aldrich). Six triangular sticky traps baited with aldehydes were set apart from each other (ca 4 m interval) without positional change in the same mushroom house. Trapped flies were counted and categorized under a binocular microscope one week after the distributed traps were collected back. (The first two lures were duplicated but others were not due to grower convenience and limited space).

### Results

Table 5-3-1 summarizes trap catches of three types of aldehyde lures and a control. Compared to the control, male *B. paupera* flies captured by aldehyde C11 and mixtures of C10-C13 lures were significantly higher, but no sexual differences were observed in trap catches of *L. mali* (Chi- square test, $p<0.05$). The mixture of C7-C18 it was not compared with the others because only one trap was set. Preliminary field test results demonstrated the aldehyde mixture or undecanal

alone had the highest attractive activity in mushroom houses. More data is needed for detailed analysis and confirmation.

Fig. 5-3-1. A photograph showing sticky trap setting in field mushroom trapping test.

Table 5-3-1. Field trap catches of two species of darkwinged fungus gnats in a mushroom cellar.

| Lure | Amount | Ratio | *B. paupera* | | | *L. mali* | | |
|---|---|---|---|---|---|---|---|---|
| | | | Male | | Female | Male | | Female |
| Undecanal | 1 mg | — | 15.0 | * | 5.0 | 47.5 | ns | 46.5 |
| Ald: C10-C13 | 1mg | Natural | 14.8 | * | 5.6 | 58.0 | ns | 34.5 |
| Ald: C7-C18 | 1mg | Equal | 17 | * | 8 | 45 | ns | 32 |
| Control Hexane | 10 ?l | — | 8 | ns | 6 | 28 | ns | 16 |

* Significantly different as tested by chi-square test at P< 0.05, ns. Not significantly different

**Discussion**

Preliminary field test results showed that 1-undecanal alone, a *n*-ald:C10-C13 mixture and a *n*-ald:C7-C18 mixture significantly trapped more males *B. paupera* than females. However, no choice preference choice was observed between males

and females of *L. mali*. Capture of numbers of both sexes of *L. mali* reflected high population numbers of this species in the mushroom house. Black light traps also predominantly attracted catches of *L. mali* (unpubished data). However, *B. paupera* females were also in the black light catches, but were half or one third of the catches of *L. mali*. According to the results of light trapping using a black UV lamp (Ishitani et al., 1997), 19 times more female (2,080) were caught than males' (152), thus more male *B. paupera* being trapped by *n*-aldehyde can be considered a real effect. However, it more tests are needed to clarify this effect.

## Chapter VI. General Discussion

Since there are few reports relative to the chemoecology of the fungus gnat, the results obtained in this study demonstrated interesting biological characteristic of *B. paupera*, e.g. female reproduce unisexual offspring and release volatile sex pheromone from the body surface at the later pupal stage.

The reason for reproduction of unisexual offspring was analyzed as occurring because of the elimination of one or two X chromosomes at the seventh or eighth cleavage of the zygote cells (Metz, 1938). If one chromosome is eliminated, the somatic cells will be X'X and the individual will develop into a female. If two are eliminated, the soma will be XO and result in development of a male. *Bradysia* genus possessing both a 1V/3R chromosome complement that results in a monogenous speices and a 2V/2R chromosome complement which induces a digenic species. The speices in the closely-related genus *Lycoriella* contains a 2V/2R chromosome complement, thus all species exhibit digeny. It has been suggested that monogeny is derived and digeny is the ancestral trait (Harris et al. 1996). Concerning the significance of some *Bradysia* producing unisexual offspring, Metz (1938) suggested the production of unisexual progeny is a means of insuring out crossing.

From the post pupal stage, female *B. paupera* are able to produce sex pheromone on the body surface. Sex pheromones have merely referred to in other reports, this study shows this species shares the same biosynthetic pathway for a cuticular sex pheromone that has been proposed and partially demonstrated in other dipterans. Among the Diptera, cuticular hydrocarbon associated sex pheromone biosynthesis has been extensively studied in the higher flies (suborder Brachycera) (Tillman et al., 1999). Hydrocarbon based dipteran pheromone compounds have been demonstrated in the cuticle and shown to be structurally

similar to components in the epicuticular lipid layer of all insects (Blomquist et al., 1998). These pheromone components are synthesized by modifications in the pathways that produce cuticular lipids (Blomquist et al., 1987; Nelson and Blomquist, 1995). The epoxide and ketone pheromone components of *M. domestica*, *Z*9,10-23:epoxide and *Z*14-23:ketone, appear on the female cuticle simultaneously with *Z*9-23:hydrocarbon (Blomquist et al., 1984). Labeled *Z*9,10-23:epoxide and *Z*14-23:ketone were isolated from females subsequent to topical treatment with [9,10-3 H] *Z*9-23:HC, demonstrating that *Z*9-23:HC is converted to these oxygenated derivatives. Oxidase enzymes play a critical role in the oxidation of Z9-23:HC to the corresponding epoxide and ketone. Several studies indicate that hydrocarbons are synthesized by oenocytes widely occurring in insects, even in insects whose oenocytes are located within the epidermis. It appears that hydrocarbons are carried through the hemolymph to the epicuticle and other destinations, and lipophorin acts as the shuttle (Pho et. al., 1996). Wicker-Thomas et al. (1997) cloned and partially characterized a desaturase gene from *D. melanogaster* that appears to be expressed more in females than males, suggesting a role for the gene product in the first desaturation step of *D. melanogaster* pheromone biosynthesis. In a particularly exciting approach, the site of synthesis and genetic basis for 7-monoenes and 7,11-dienes were explored in male *D. melanogaster* that had been feminized by using a gene transformer (Ferveur et al., 1997). This gene appears to be a master regulator initiating the synthesis of sexually dimorphic hydrocarbons in the oenocytes, early in the life of the imago. Female *B. paupera* elicit male behavioral responses immediately after elusion indicating a similar mechanism.

As the only identified sex pheromone in the darkwinged fungus gnat (Kostelc, et al., 1979) was reported to contain standard hydrocarbons C15-C26 to which *L. mali* males responded with sex pheromonal activity. The Heptadecane was shown to be a major attractant at 1 µg - 1 ng. However the behavioral indicators e g. WF, ZA and LO were not presented. Hydrocarbons C11-C13 are also observed behaviorally responded by male *B. paupera* with the strongest response to C12. However this response rate was lower than to undecanal. *L. mali* and *B. paupera* are reproductively isolated symbiotic species. However, at present there is still no data to compare the sex pheromonal component of the two species. In the list of identified sex pheromones for dipterans, most pheromone are composed of unsaturated hydrocarbons such as *Drosophila melanogaster* (Antony et al., 1985), *D. pallidosa* (Nemoto et al., 1994) *Musca domestica* (Carlson et al., 1971) or branched saturated hydrocarbons, such as *Glossina morsitans* (Carlson and Schlein, 1991) *Culicoides melleuss* (Linley and Carlson, 1978) *Agromyza frontella* (Carrie et al., 1988).

The 2-ketones occupied 44% of the cuticular lipids of *B. paupera*, but no behavioral activity was demonstrated. Whether these 2-ketones involved in the process of the biosynthesis of the sex pheromone component remains unclear. However, ketones have been reported as aggregation pheromones in *Drosophila hydei* (Moats et al., 1987), *D. martensis* (Schaner and Jackson, 1992), *D. serido* (Schaner and Jackson, 1992).

As aldehydes functioning as semiochemicals in Diptera, only hexanal as a sex pheromone elute from adult males and attractive to females have been shown in the flesh fly *Sarcophaga bullata* (Parker) (Gerard et al., 1979). A series of aldehydes existence in the cuticular extracts of *B. paupera* and behaviorally active to males, is therefore characteristic and interesting, but these aldehydes is not likely the main sex pheromone because the their attractive activities are still weak and responses of the GC-EAD to crude female extracts indicate certain components more electrophysiological active. Thus to unveil the complexity of the sex pheromone further studies and precise analysis, especially behavioral combined diagnostic (Howse, 1998) analyses are needed.

This study appears to be the first time stable and clear EAD recordings for a tender small in such tender small antenna (0.6-0.9 mm) have been obtained. By the use of a custom-designed apparatus, characteristic responses were measured from a series synthetics chemicals which had been identified from the cuticular extract of *B. paupera* with an antennae preparation. The intensities of the electrophysiological responses to the chemicals reflected well the behavioral examination. Nojima et al. (2003) elaborately mounted a cut head of male *Rhgoletis pomonella* on a acrylic holder and acquired excellent EAD recordings for a few hours. The EAD electrode developed here achieved similar effectiveness with a simpler shape. It is hoped to be useful for electrophysiological measurement in other sciarid flies.

Based on highest EAG responses obtained to 12-carbon atom compounds, acetate functional groups and Z isomers, Roelofs and Carde (1974) used a series of clues to deduce successfully the structure of the sexual pheromone of the lesser apple worm moth, *Grapholitha prunivora*. Preliminary EAD testing of chemicals of a female origin by male *B. paupera* antennae concisely showed highest responses to 11-carbon atom compounds and to aldehyde functional groups. Also these responses corresponded to the behavioral bioassay, but the sex pheromone structure for *B. paupera* cannot be concluded on the present data because of muti-component showed sex pheromonal activity and remains 3 EAD active components unidentified.

Behaviors of the *B. paupera* male can be affected by intensities of illumination.

When switching to dark conditions, flight behavior but not walking showed significantly different to the light conditions. Particular light intensity can trigger or inhibit sexual behaviour of some insect species (Shorey, 1974), therefore, the effect of illumination on pheromone application should be consider for better trapping method with attractant e.g. sex pheromone or the case accompanying fluorescent lamp.

Field test conducted with 1-undecanal and aldehydes mixture described in section 3 chapter V is a preliminary test. Significantly more male *B. paupera* than females have been trapped means aldehydes compounds is effective in the field condition although more data are needed to prove the feasibility of the method and attractant. Because of the female sex pheromone evoked strong attraction and mating behavior to male flies that the expectancy to develop a sex pheromonal control option is considered realizable.

*B. paupera* male responded behaviorally to *n*-aldehyde of C10-C13, *n*-Hydrocarbon of C10-C12 and other chemicals structurally similar to undecanal. However, do insects have the capability to utilize all of this potential information ? As we look deeper into the organizational details of the glomerular neuropil, however, we can see remarkable similarities in the structure and function of the synaptic circuits that process two types of information. This suggests the current state of knowledge in this field is premature to regard the pheromone-processing subsystem as a mechanistically distinct, 'special case' in olfaction. In the absence of bioassays of the individual chemicals we have no way to answer this question. Yet little effort has been made to elucidate with the genetic basis of semiochemical and neurobiological roles of the information processed in the insect at the peripheral receptor level or in the central nervous system. But with increasing progress and the ready availability of instrumentation that is more sophisticated and sensitive we enter into the phase of looking at the evolutionary origins of semiochemical functions.

The results obtained in this study only serve as part of the answers to provide better understanding of fungus gnat biology.

## Summary

From the chemoecological studies in this paper, the acquired knowledge related to the reproductive behaviors of the darkwinged fungus gnat, *Bradysia paupera*, can be summarized as follows:

1. Rearing single pregnant females clarified that this species reproduces

unisexual offspring and has a preference for fungus as food.

2. Mating behaviors of *B. paupera* consisted of a series of stereotyped male behaviors including intermittent wing fanning (WF), walking with a zigzag approach (ZA) while continuing WF, abdomen curling (AC) forward beneath the thorax while using his claspers to grab the female abdomen, pivoting 180 degrees around body axis and mating occurrence (MO).
3. Female flies emit volatile sex pheromone from the body surface from the later pupal stage until death. Female cuticular sex pheromone evokes a series of copulating behavior and causes strong chemotaxis flight in males irrelative of the dial periodicity.
4. Male flies begin sexual responses 30 min after adult eclosion and response increase to a maximum at 2 hr after emergence.
5. Low light intensity depresses male flight activity, but not other locomotor activity, low illumination has no affect on close range mating behavior.
6. Properly setting of antenna preparations and use of the correct saline allows for recording of electrophysiological responses from tiny antennae of male *B. paupera* based on ordinary amplifier facilities.
7. *n*-Aldehydes of C6-C18, 2-ketones of C16-C21 and *n*-hydrocarbons of C10-C26 were identified from female cuticular lipids. 2-ketone of C16-C21 and *n*-aldehydes of C6-C9 are also common in male cuticular lipids.
8. Male antenna responded with comparatively more sensitivity to *n*-aldehydes than 2-ketones and *n*-hydrocarbons. In *n*-hydrocarbons males were more sensitive to carbon chains of C11-C12 than longer or shorter ones. Female antennae were more sensitive to food related compounds than extracts of a mate.
9. *n*-Aldehydes of C10-C13, *n*-hydrocarbons of C11-C13 and other chemicals with structure similar to undecanal elicited typical sex pheromonal behaviors in males. Among these substances undecanal was the most active component.
10. An *n*-undecanal and *n*-aldehyde mixture trapped significantly more males than females in preliminary field tests.
11. Trace amounts of EAD active components important for sex pheromonal activity remain to be identified.

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## References

Alberts, S. A., M. K. Kennedy and R. T. Carde (1981) Pheromone mediated anemotactic flight and mating behavior of the sciarid fly *Bradysia impatiense*. *Eviron. Entomol.* 10: 10-15.

Antony, C., T. L. Davis, D. A. Carlson, J. M. Pechine and J. M. Jallon (1985) Compared behavioral responses of male *Drosophila melanogasster* (Canton S) to natural and synthetic aphrodisiacs. *J. Chem. Ecol.* 11:1617-1629.

Ausloos, P., C. L. Clifton, S. G. Lias, A. I. Mikaya, S. E. Stein, D. V. Tchekhovskoi, O. D. Sparkman, V. Zaikin and D. Zhu (1999) The critical evaluation of comprehensive mass spectral library. *J. Amer. Soc. Mass Spectrom.* 10: 287-299.

Baker, T. C. and R. T. Carde (1984) Techniques of behavioral bioassay. In: *Techniques in Pheromone Research* (H. E. Hummel and T. A. Miller eds.). Springer-Verlag, New York, pp. 45-73.

Bartlett, G. R. and C. B. O. Keil (1997) Identification and characterization of a permethrin resistance mechanism in population of the fungus gnat *Lycoriella mali* (Fetch).(Diptera: Sciaridae). *Pestic. Biochem. Physiol.* 58: 173-181.

Berenbaum, M. R. (2002) Pheromonal and host-odor processing in the insect antennal lobe: how different?. *Curr. Opin. Neurobiol.* 12: 393-399.

Blomquist, G. J. T. S. Adams and J. W. Dillwith (1984a) Induction of female sex pheromone production in male houseflies by ovarian implants or 20-hydroxyecdysone. *J. Insect Physiol.* 30: 295-302.

Blomquist, G. J., J. W. Dillwith and J. G. Pomonis (1984b) Sex pheromone of the housefly: metabolism of (Z)-9-tricosene to (Z)-9,10-epoxy-tricosane and (Z)-14-tricosen-10-one. *Insect Biochem.* 14: 279-284.

Blomquist, G. J., J. W. Dillwith and T. S. Adams (1987a) Biosynthesis and endocrine regulation of sex pheromone production in Diptera. In: *Pheromone Biochemistry*. (G. J. Blomquist and G. D. Prestwich eds.). Academic Press, Orlando, Florida, pp. 217-250.

Blomquist, G. J., D. R. Nelson and M. DeRenobales (1987b) Chemistry, biochemistry, and physiology of insect cuticular lipids. *Arch. Insect Biochem. Physiol.* 6: 227-265.

Blomquist, G. J., C. E. Borgeson and M. Vundla (1991) Polyunsaturated fatty acids and eicosanoids in insects. *Insect Biochem.* 21: 99-106.

Blomquist, G. J., J. A. Tillman-Wall, L. Guo, D. R. Quilici and P. Gu (1993) Hydrocarbon and hydrocarbon derived sex pheromones in insects: biochemistry and endocrine regulation. In: *Insect Lipids: Chemistry, Biochemistry, and Biology*. (D. W. Stanley-Samuelson, D. R. Nelson eds.). University of Nebraska Press, Lincoln, Nebraska, pp. 318-351.

Blomquist, G. J., L. Guo, P. Gu, C. Blomquist, R. C. Reitz and J. R. Reed (1994) Methyl-branched fatty acids and their biosynthesis in the housefly, *Musca domestica* L. (Diptera: Muscidae). *Insect Biochem. Mol. Biol.* 24: 803–810.

Brewer, K. K. (1990) Stability of adaptation to permethrin in a laboratory study with the mushroom sciarid *Lycoriella mali* (Fitch) (Diptera: Sciaridae). *Mushroom News.* 27: 230-238.

Brar, D. S. and G. S. Sandhu (1989) Biology of the sciarid fly, *Bradysia tritici* (Coq.) (Diptera: Sciaridae) on temperate mushroom in the Punjab (India). *Indian J. Entomol.* 49:267-274.

Brinton, F. E., M. D. Proverbs, and B. E. Carty (1969) Artificial diet for mass production of the codling moth, *Carpocapsa pomonella* (Lepidoptera: Olethreutidae). *Can. Entomol.* 101: 577-584.

Cantelo, W. W. (1989) Advances in control of the sciarid fly *Lycoriella mali* (Fitch). *Mushroom Science* 11: 255-264.

Carlson, D. A., M. S. Mayer, D. L. Silhacek, J. D. James, M. Beroza and B. A. Bierl (1971) Sex attractant pheromone of the house fly: isolation, identification and synthesis. *Science* 174: 76-78.

Carlson, D. A. and Y. Schlein (1991) Unusual polymethyl alkene in tsetse flies acting as abstinon in *Glossina morsitans*. *J. Chem. Ecol.* 17: 267-284.

Carrie, Y., J. G. Millar, J. N. McNeil, D. Miller and E. W. Underhill (1988) Identification of female sex pheromone in alfalfa blotch leafminer, *Agromyza frontella* (Ronidani) (Diptera: Agromyzidae). *J. Chem. Ecol.* 14: 947-956.

Christensen T. A, V. M. Pawlowski, H. Lei and J. G. Hildebrand (2000) Multi-unit recordings reveal context-dependent modulation of synchrony in odor-specific neural ensembles. *Nat. Neurosci.* 3: 927-931.

Corey, E. J. and G. Schmidt (1979) Useful procedures for the oxidation of alcohols involving pyridinium dichromate in aprotic media. *Tetrahedron Lett.* 5: 399-402.

Coyne, J. A. and R. Oyama (1995) Localization of pheromonal sexual dimorphism in *Drosophica melanogaster* and its effect on sexual isolation. *Proc. Natl. Acad. Sci. USA.* 92: 9505-9509.

Dillwith, J. W. and G. J. Blomquist (1982) Site of sex pheromone biosynthesis in the female housefly, *Musca domestica* L. *Experientia* 38: 471-473.

Ferveur J. F., F. Savarit, C. J. O'Kane, G. Sureau, R. J. Greenspan and J. M. Jallon (1997) Genetic feminization of pheromones and its behavioral consequences in *Drosophila* males. *Science* 276: 1555-1558.

Finley, R. J., P. J. Wuest, D. J. Royse, R. Snetsinger, R. Tetrault and D. L. Rinker (1984) Mushroom flies - A review of biology and a systems approach for integrated control. Mushroom News 28: 240-246.

Gardiner, R. B., W. R. Jarvis, J. L. Shipp (1990) Ingestion of *Pythium spp*. by larvae of the fungus gnat *Bradysia impatiens* (Diptera: Sciaridae). *Ann. Appl. Biol.* 116: 205-212.

Girard, J. E., F. J. Germino, J. P. Budris, R. A. Vita and M. P. Garrity (1979) Pheromone of the male flesh fly, *Sarcophaga bullata. J. Chem. Ecol.* 5: 125-130.

Gouge, D. H. and N. G. M. Hague (1994) Glasshouse control of fungus gnats,

*Bradysia paupera* on fuchsias by *Steinernema feltiae*. *Fund*. *Appl*. *Nematol*. 18: 77-80.

Harris, M. A, W. A. Gardner and R. D. Oetting (1996) A review of the scientific literature on fungus gnats (Diptera: Sciaridae) in the genus *Bradysia*. *J*. *Entomol*. *Sci*. 31: 252-276.

Heath, R. R. and J. H. Humlinson, (1984) Techniques for purifying, analyzing, and identifying pheromones. In: *Techniques in pheromone research* (H. E. Hummel and T. A. Miller eds). Springer-Verlag, New York, pp. 287-322.

Hellqvist, S. (1994) Biology of *Synacra* sp. (Hym., Diapriidae) a parasitoid of *Bradysia paupera* (Dipt., Sciaridae) in Swedish greenhouses. *J*. *Appl*. *Entomol*. 117: 491-497.

Himeno, K. and H. Honda (1992) (*E, Z*)- and (*E, E*)-10, 12-Hexadecadienals, major components of female sex pheromone of the cotton leaf-roller *Notarcha derogata* (Fabricius) (Lepidoptera: Pyralidae). *Appl. Entomol. Zool*. 27: 507-515.

Honda, H., J. I. Kaneko, Y. Konno and Y. Matsumoto (1979) A simple method for mass-rearing of the yellow peach moth, *Dichocrocis punctiferalis* Guenee (Lepidoptera: Pyralidae), on an artificial diet. *Appl. Entomol. Zool*. 14: 464-468.

Howse, E. P. (1998) Pheromones and behaviour. In: *Insect pheromones and their use in pest manegement*. (P. Howse, I. Stevens and O. Jones eds.). Chapman & Hall, Tokyo, pp. 3-37.

Howard, R. W. (1993) Cuticular hydrocarbons and chemical communication. In: *Insect lipids: Chemistry, Biochemistry and Biology*. (D. W. Stanley-Samuelson and D. R. Nelson eds.).University of Nebraska Press, Lincoln, Nebraska, pp. 179-226.

Howard, R. W., L. L. Jackson, H. Banse and M. W. Blows (2003) Cuticular hydrocarbons of *Drosophila birchii* and *D. serrata*: Identification and role in mate choice in *D. serrata*. *J. Chem. Ecol*. 29: 961-976.

Hudson, E. K. (1974) Regulation of greenhouse sciarid fly populations using *Tetradonema plicans* (Nematoda: Mermithoidea). *J. Invertebr. Pathol*. 23: 85-91.

Ishitani, E., K. Niijima and M. Ito (1993) Damage of mushrooms (*Agaricus bisporus* Oilat) attacked by *Lycoriella mali* (Fitch) (Sciaridae, Diptera) in Chiba prefecture. *J. Jpn For. Soc. Kanto*. 44: 175-176.

Ishitani, E., T. Gotoh and T. Kawasaki (1997) Development of sticky light trap and attractiveness to mushroom infesting sciaridys, *Lycoriella mali* (Fitch) and

*Bradysia paupera* Tuomikoski (Diptera: Sciaridae). *Jpn. J. Appl. Entomol. Zool*. 41: 141-146. (In Japanese with English summary).

Ismai, M. T and D. A. Carlson (1983) Determination of the site of pheromone emission in the virgin female *culicoides nubecalosus* Meigen. *J. Insect Physiol.* 29: 221-224.

Jarvis, W. R., J. L. Shipp and R. B. Gardiner (1993) Transmission of *Pythium aphanidermatum* to greenhouse cucumber by the fungus gnat, *Bradysia impatiens* (Diptera: Sciaridae). *Annal. Appl. Biol*. 122: 23-29.

Jess, S. and M. Kilpatrick (2000) An integrated approach to the control of *Lycoriella solani* (Diptera:Sciaridae) during production of the cultivated mushroom (*Agaricus bisporus*). *Pest Manag. Sci.* 56: 477-485.

Jones, O. T., J. C Lisk,. C. Longhurst, P. E. Howse, P. Ramos and M. Campos (1983) Development of a monitoring trap for the olive fly, *Dacus oleae* (Gmelin) (Diptera: Tephritidae), using a component of its sex pheromone as lure. *Bull. Entomol Res*. 73: 97-106.

Jones, O. T. (1998) Practical applications of pheromones and other semiochemicals. In: *Insect pheromones and their use in pest manegement*. (P. Howse, I. Stevens and O. Jones eds.). Chapman & Hall, Tokyo, pp.263-351.

Kaissling, K. E., and J. Thorson (1980) Insect olfactory sensilla: structural, chemical and electrical aspects of the functional organization. In: *Receptors for Neurotransmitters, Hormones and Pheromones in Insects*. (D. B. Sattelle, L. M. Hall and J. G. Hildebrand eds.). Elsevire/North-Holland, Biomedical Press, New York, pp. 13-55.

Kalb, D. W. and R. L. Millar (1986) Dispersal of *Verticillium alboatrum* by the fungus gnat (*Bradysia impatiens*). *Plant Dis.* 70:752-753.

Kanmiya, K. (1999) Acoustic communication in insect. In: *Enveromental Entomology- Behavior, Physiology and Chemical Ecology*. (K. Honda and H. Honda, eds.). Uneversity of Tokyo Press, Tokyo, pp. 495-509. (In Japanese)

Kennedy, M. K. (1973) A culture method for *Bradysia impatiens* (Diptera: Sciaridae). *Ann. Entomol. Soc. Am.* 66: 1163-1164.

Kennedy, M. K. (1974) Survival and development of *Bradysia impatiens* (Diptera: Sciaridae) on fungal and non-fungal food sources. *Ann. Entomol. Soc. Am.* 67: 745-749.

King, A. I. (1991) Efficacy of insect growth regulators for fungus gnat larvae control in potted foliage plants. *HortScience* 26: 708-713.

Kostelc, J. G., B. J. Garcia, G. W. Gokel and L. B. Hendry (1979) Macrocyclic polyethers as probes into pheromone receptor mechanisms of a sciarid fly

*Lycoriella mali* Fitch. *J. Chem. Ecol*. 5: 179-185.

Kostelc, J. G., J. E. Girard and L. B. Hendry (1980) Isolation and identification of a sex attractant of a mushroom infesting sciarid fly. *J. Chem. Ecol*. 6: 1-11.

Landolt, P. J., R. R. Heath, H. R. Agee, J. H. Tumlinson and C. O Calkins. (1988) Sex pheromone-based trapping system for papaya fruit fly (Diptera: Tephritidae). *J. Econ. Entomol.* 81: 1163-1169.

Langley, P. A and D. A. Carlson (1983) Biosynthesis of contact sex pheromone in the female tsetse fly, *Glossina Morsitans* West-wood. *J. Insect Physiol*. 29: 825-831.

Linley, J. R. and D. A. Carlson (1978) A contact mating pheromone in the biting midge, *Culicoides melleus*. *J. Insect Physiol*. 24: 423-427.

Liu, Y., H. Honda and Y. Kono (2002) Mating behavior and its regulatory factors in the black fungus gnat, *Bradysia paupera* (Diptera: Sciaridae). *Appl. Entomol. Zool*. 46: 23-30. (In Japanese with English summary).

Malo, E. A., M. Renou and A. Guerrero (2000) Analytical studies of *Spodoptera littoralis* sex pheromone components by electroantennography and coupled gas chromatography-electroantennographic dectetion. *Talanta* 52: 525-532.

Mayer, M. S. and J. R. McLanghlin (1991) *Handbook of Insect Pheromones and Sex Attractants*. CRC Press, Boca Raton, pp.1083.

Metz, C. W. (1938) Chromosome behavior inheritance and sex determination in *Sciara. Am. Natur*. 72: 485-520.

Michel, R. and G. Angel (2000) Insect parapheromones in olfaction research and semiochemicals based pest control strategies, *Annu. Rev. Entomol.* 45: 605-630.

Moats, R. A., R. J. Bartelt, L. L. Jackson and A. M. Schaner (1987) Ester and ketone components of aggregation pheromone of *Drosophila hydei* (Diptera: Drosophilidae). *J. Chem. Ecol*. 13: 451-462.

Moore, I. (1981) Biological amplification for increasing electroantennogram discrimination between two female sex pheromones of *Spodopetera lottoralis* (Lepidoptera: Noctuidae). *J. Chem. Ecol*. 7:791-798.

Nemoto, T., M. Doi, K. Oshio, H. Mtsubayashi, Y. Oguma, T. Suzuki and Y. Kuwahara (1994) (Z, Z)-5, 27-tritriacontadiene: major sex pheromone of *Drosophila pallidosa* (Diptera: Drosophilidae). *J. Chem. Ecol*. 20: 3029-3037.

Nojima, S., L. J. Charles, B. Morris, A. Zhang, and W. Roelofs (2003) Identification of host fruit volatiles from hawthorn (Crataegus spp.) attractive to hawthorm-origin *Rhagoletis pomonella* flies. *J. Chem. Ecol*. 29: 321-336.

Oguma, Y., T. Nemoto and Y. Kuwahara (1992) A sex pheromone study of a fruit fly

*Drosophila virilis* Sturtevant (Diptera: Drosophilidae): additive effect of cuticular alkadienes to the major sex pheromone. *Appl. Entomol. Zool*. 27: 499-505.

Osborne, L. S., D. G. Boucias and R. K. Lindquist (1985) Activity of *Bacillus thuringiensis* ver. *Israelensis* on *Bradysia coprophila* (Diptera: Sciaridae). *J. Econ. Entomol*. 78: 922-925.

Percy-Cunningham, J. E. and J. A. MacDonald (1987) Biology and ultra-structure of sex pheromone producing glands. In: *Pheromone Biochemistry* (G. J. Blomquist and G. D. prestwich eds.). Academic Press, Orlando, Florida, pp. 27-75.

Pho, D. B., M. Pennanech and J. M. Jallon (1996) Purification of adult *Drosophila melanogaster* lipophorin and its role in hydrocarbon transport. *Arch. Insect Biochem. Physiol*. 31: 289-303.

Ponomarev, V. L., N. V. Vendilo, V. A. Pletnev, K. V. Lebedeva and N. N. Melnikov (1997) Influence of diet upon the composition of pheromone volatiles in *Galleria mellonella* (greater wax moth) males. *Arch. Insect Biochem. Physiol*. 36: 129-138.

Rakowski, G. (1988) Effect of illumination intensity on the response of the hide beetle, *Dermestes maculates*, to aggregation pheromone. *J. Insect Physiol*. 34: 1101-1104.

Roelofs, W. L. (1984) Electroantennogram Assay: rapid and convenient screening procedures for pheromones. In *Techniques in pheromone research*. (H. E. Hummel and T. A. Miller eds.). Springer-Verlag, New York, pp.131-159.

Roelofs, W. L. and R. T. Carde (1974) Oriental fruit moth and lesser appleworm attractant mixtures redefined. *Environ. Ent*. 3:586-588.

Sasakawa, M. (1985) Agricultural crops infested sciarid flies. *J. Jpn. Crop. Prot*. 29: 56-60. (In Japanese)

Sasakawa, M. (1993) Japanese mushroom gnats (Diptera: Sciaridae). *Jpn. J. Environ. Entomol. Zool*. 5: 1-5. (In Japanese)

Sato, Y. and K. Asawa (1995) Uptake of the insectiside, fenitrothion, by fruit bodies of shiitake, (*Lentinus edodes*) from treated bed logs. *J. Jpn. For. Soc*. 77: 220-223. (In Japanese)

Sato, Y., A. Sekiya and K. Asawa (1995) The uptake of the fungicide, thiabendazole by the oyster mushroom, (*Pleurotus ostreatus)* cultivated on a sawdust medium. *J. Jpn. For. Soc*. 77: 353-357. (In Japanese)

Schaner, A. M. and L. L. Jackson (1992) (Z)-10-Heptadecen-2-one and other 2-ketones in the aggregation pheromone blend of *Drosophila martensis*, *D.*

*buzatii*, and D. *srido*. *J. Chem. Ecol*. 18: 53-64.

Shorey, H. H. (1974) Environmental and physiological control of insect sex pheromone behaviour. In: *Pheromones*. (M. Brirch ed.). Noth-Holland Elsevier, Amesterdam, pp. 62-80.

Shields V. D. C and J. G. Hildebrand (2001) Responses of a population of antennal olfactory receptor cells in the female moth *Manduca sexta* to plant-associated volatile organic compounds. *J. Comp. Physiol*. 186: 1135-1151.

Silerstein, R. M., C. G. Basslar and T. C. Morrill (1981) *Spectrometric Identification of Organic Compounds*. John Wiley & Sons, Inc. New York, pp.1-40

Steffan, W. A. (1996) A generic revision of the family Sciaridae (Diptera) of America nother of Mexico . *Univ. of Calif. Publ. Entomol*. 44: 1-77.

Stevens, I. D. R. (1998) Chemical aspects of pheromone in *Insect Pheromones and their Use in Pest Management*. (P. Howse, I. Stevens and O. Jones eds). Chapman&Hall, Tokyo, pp. 135-260.

Struble, D. L. and H. Arn, (1984) Combined gas chromatography and electroantennogram recording of insect olfactory responses In *Techniques in pheromone research* (H. E. Hummel and T. A. Miller eds), Springer-Verlag, New York, pp. 161-189.

Suzuki, Y., Y. Saito, M. Toyota (1990) *Thin Layer Chromatography*. Hirokawa Shoten, Tokyo, pp. 9-74. (In Japanese)

Tadao, G., K. Nakamuda, M. Tokoro and T. Nakashima (1999) Copulatory behavior and sex pheromone in sciarid fly, *Lycoriella mali* (Fitch) (Sciaridae: Diptera). *Jpn. J. Appl. Entomol. Zool*. 43: 181-184. (In Japanese with English summary).

Tillman, J. A., S. J. Seybold, R. A. Jurenka and G. J. Blomquist (1999) Insect pheromones-an overview of biosynthesis and endocrine regulation. *Insect Biochem. Mol. Biol*. 29: 481-514.

Wicker-Thomas, C., C. Henriet and R. Dallerac (1997) Partial characterization of a fatty acid desaturase gene in *Drosophila melanogaster*. *Insect Biochem. Molec. Biol*. 27: 963-972.

Wright, E. M. and R. J. Chambers (1994) The biology of the predatory mite *Hypoaspis miles* (Acari: Laelapidae) a potential biological control agent of *Bradysia paupera* (Diptera: Sciaredae). *Entomophaga* 39: 225-235.

Ydergaard, S., A. Enkegaad and H. F. Brodsgaard (1997) The predatory mite *Hypoaspis miles* : Temperature dependent life table characteristics on a diet of sciarid lavae *Bradysia paupera* and *B. tritici*. *Ent. Exp. Appl*. 85: 177-187.

Yoshiga, T., N. Yokoyama, N. Imai, A. Ohnishi, K. Moto and S. Matsumoto (2002) cDNA cloning of calcineurin heterosubunits from the pheromone gland of the silkmoth, *Bombyx mori*. *Insect Biochem. Molec. Biol*. 32: 477-486.