

CHAPTER 4

DISCUSSION

In aiming to search for the main attractive ingredients and develop effective olfactory-related bait, using natural products as a prototype to lure the medically important blow fly, *C. megacephala*, laboratory and field investigations on behavioral response of this fly species to various animal-origin baits were revealed. The effectiveness of bait not only involves the chemical cues provided by olfactory-related bait, but also various physiological factors of the flies themselves (Sucharit et al. 1976, Spradbery 1979, Chaudhury et al. 2002, Davidson et al. 2006), including age, sex, reproductive profile (mated vs virgin), etc. Abiotic environmental factors such as wind speed, light intensity, temperature, and relative humidity are, to a degree, associated with such effectiveness (Cardé 1996, Geier et al. 1999, Marchand and McNeil 2000).

Ideally, an electrophysiological technique has proved to be a valuable tool for investigating olfactory attractant in insects, based on its rapid detection measured by electrical change across the whole antenna or single sensory cell. As for the blow fly, Park and Cork (1999) used an electroantennogram to investigate specific attractant from a mammalian body and excreta to lure *Lucillia* spp. However, the reflection of antenna response to compounds by this technique cannot provide real information of insect behavioral response (Ridgway et al. 1990, Tumlinson 1990). Investigation by Williams et al. (2010) revealed that insect did not only use a single sensory cell or an

antenna for responding to some stimulants, but also various cells that they operate to express their behavior responses. Among the behavior bioassays currently available, wind tunnel is the most useful method in which enough space for free-flying and airflow source for odor distribution is provided.

A variety of wind tunnels have been designed and employed to investigate the behavioral responses of several different insect species (Geier et al. 1999, Burkett and Butler 2005, Okada and Sakuma 2009, Brevault and Quilici 2010). To test blow flies, Bunchu et al. (2008) constructed and operated a “T-box dual-choice wind tunnel” to investigate the behavioral response of *C. megacephala*. This model has the advantage of a dispersing odor system that is like the natural environment, in which flies generally use positive anemotaxis for navigation towards the odor source. However, limitations in this model were detected during experimental procedures such as 1) its size is large (60×60×160 cm), which makes it difficult to move or clean, 2) it is constructed in acrylic-coated plywood, which can absorb the tested odor that remains after the experiment, 3) its fan produces the highest wind speed limited at 0.5 m/s, and 4) its experiments are conducted in a room, without residual odor eliminators. Thus, a new wind tunnel model (30×30×250 cm) has been designed and constructed to rectify these and other drawbacks, and improve the efficacy of testing the behavioral response of this fly species. The primary components of the wind tunnel include the stimulant compartments, trap compartments and a release compartment are made from non-odor retained materials of clear glass sheets held within a lightweight aluminum framework (Tholl et al. 2006). Each compartment can be separated independently from the wind tunnel, thus allowing easy cleaning and removal of any trapped odors from previous experimental trials. In addition, the clear compartments

are conjoined in the middle of the tunnel, which allows researchers to observe visually the flies in each one. Adjustment of wind speeds is accomplished by using various options in the electric potential of the tunnel fans. The use of new fans to improve the wind tunnel has attained a high wind speed of 0.93 m/s, instead of the 0.5 m/s obtained in the previous model. In order to remove odor contamination from previous experiments in the wind tunnel, the rear wall of the release compartment acts as an air outlet that is connected directly to a room-ventilated fan, which expels odor emitted from bait out of the room. Furthermore, the use of vertical-room and horizontal-tunnel light allows fine control of overall light in order to achieve the appropriate light intensity for testing within the wind tunnel.

Some behavioral responses of *C. megacephala* have been worked on previously in field research experiments, which revealed that these flies respond predominantly to baits composed of protein rather than any other substances (Sucharit et al. 1976, Boonchu et al. 2003). In laboratory evaluation of this fly species, Bunchu et al. (2008) indicated that 1-day tainted pork scrap was the most attractive bait to *C. megacephala*, which agrees with the findings of this study. This explanation relies on the basic biology that the fly itself needs to obtain essential nutrients from feeding substrates to live. In adults, protein content in food is a requirement for reproductive maturation and succession. Protein has been found to activate the development of accessory glands and the neuroendocrine system in males of the blow fly, *Phormia regina*, thus enhancing their mating behavior; whereas, protein increases oogenesis in females of *P. regina* (Stoffolano et al. 1995), *C. megacephala*, and *Chrysomya putoria* (Linhares and Avancini 1989). Insufficient intake of protein results in delayed development of ovarian follicles and yolk production in females of the blow flies, *L. cuprina*, *Lucilia*

sericata and *P. regina* (Stoffolano et al. 1995, Wall et al. 2002). In these experiments, even though flies had been provided with fresh pork liver within the first three days post eclosion, adults still need a protein diet as bait to attract them in the wind tunnel. High protein content is a major component of fresh pork or animal scrap; whereas, chub mackerel contains a relatively small amount of protein (15-20%) (Adams and Moss 1995). This may explain in part the low attractive index of chub mackerel in this study. Nevertheless, *C. megacephala* is an anautogenous species, and protein plays an essential role in the development of ovarian follicles. In contrast, *L. cuprina* is an autogenous species in which females can lay eggs without protein supplementation (Spradbery 1979), although development may be delayed.

In addition to protein substrate, adult flies require food for energy to do their daily activities, and carbohydrate is a typical source for this energy. Strangways-Dixon (1962) found that adult blow fly, *Calliphora erythrocephala*, survives in nature without feeding on protein. However, they die when carbohydrate is unavailable. Results of this study demonstrated that flies starved for 24 hours respond to high attractive bait of 1-day tainted pork scrap, with no significant difference to well-fed flies. This finding agrees with results of investigations by Becher et al. (2010) who reported a non-significant difference in response to vinegar as bait between the starved and well-fed fruit fly, *Drosophila melanogaster*. Such information provides evidence of the physical adaptation of fruit flies when food supplies are limited, even though they had only been starved for a single day. However, the main purpose of the current study aimed to verify the behavioral response of well-fed flies by mimicking a typical situation found in nature, where ample food for them is available.

Blow flies, like other insects, orientate by primarily using olfactory cues, which are individual or a complex of volatile chemicals emitted from its preferred feeding and oviposition resources, at both short and long distances. From investigations in the wind tunnel, Bunchu et al. (2008) demonstrated that *C. megacephala* preferred protein-enriched products, especially 1-day tainted one that produced strong offensive odor. Other experiments in the field found that a greater number of *C. megacephala* was collected in traps baited with 1-day tainted beef scrap over those baited with fresh product (Ngoen-klan et al. 2011). In this study, results revealed that more *C. megacephala* flies were attracted to 1-tainted pork scrap than fresh pork scrap. Thus, the difference in volatile chemical profiles emitted between fresh and tainted baits caused different stimulations to *C. megacephala*. In this study, analysis through GC-MS indicated that fresh pork scrap released a variety of hydrocarbons, ammonia, carbon dioxide, elemental sulfur and propanedioic acid, which are general chemical components of raw meat (Soncin et al. 2007, Vasta et al. 2007). While the volatile composition of 1-day tainted pork scrap is characterized by many acids, ammonia, alcohols, carbon dioxide, indole, and large amounts of sulfur compounds, most of them are produced during spoilage process of meat. Dainty (1996) and Statheropoulos et al. (2007) stated the process during spoilage involves many chemical compounds, which are degraded spontaneously from carbohydrates, lipids and proteins. Mainly, volatile oxygenated compounds such as acids, alcohols, aldehydes, ketones, esters and ethers, emitted from spoilage of meat, are the products from carbohydrate degradation (Miller and Varel 2001, Statheropoulos et al. 2007). Some alcohols, aldehydes, and ketones are from lipid degradation (Mottram 1998, Machiels et al. 2004); while, several offensive-producing chemicals such as nitrogen

(ammonia, putrescine, cadaverine, trimethyl amine), sulfur (dimethyl disulfide) and phenolic (indole, skatole) compounds are degraded from protein (Chinivasagam et al. 1998, von Helmholtz 2001, Alberto et al. 2002, Kaneki et al. 2004). In addition to spontaneous autolysis, spoilage volatiles also are produced together with microbial reaction, which reacts by covering bacteria, fungi and yeasts (Nychas and Tassou 1997, Mottram 1998, Partidario et al. 2006).

Up to now, attractive chemicals have been documented from investigations into several blow fly species. As shown in Table 1.2, sulfur compounds including methyl mercaptan, dimethyl disulfide and dimethyl trisulfide act as common ingredients to lure blow flies. Otherwise, indole as well as short-chain acids and alcohols are added as minor components. In this study, volatile compounds produced from animal-origin products were characterized by using GC-MS, which is a sensitive analytical method for volatile compounds, to provide the information of attractant to *C. megacephala*. Samples were prepared by using two techniques; HS-GC-MS and HS-SPME-GC-MS. HS-GC-MS is a highly sensitive method for determining volatile compounds from various kinds of solid and liquid samples, and it detects a wide range of volatile inorganic and organic compounds. It has been used for several analyses such as those on volatile chemicals emitted from raw lamb meat (Vasta et al. 2007) and tropical prawns (Chinivasagam et al. 1998), and volatile metabolites produced from bacteria (Labows et al. 1980). HS-SPME-GC-MS is a simple and solventless technique which has been applied for many analyses such as insect sex pheromone (Bland et al. 2001, Everaerts et al. 2010, Eom et al. 2012) and decomposed human remains (Hoffman et al. 2009). By using HS-SPME-GC-MS analysis, Cosse and Baker (1996) proved that volatile compounds produced from pig manure were able to

lure *M. domestica*. Even though HS-GC-MS has the potential to detect various volatile compounds as effectively as HS-SPME-GC-MS, the SPME fiber of the latter is exposed to volatiles for a longer period during sampling collection (30 min) than the headspace technique (1 min). In addition, polydimethyl siloxane in SPME fiber has the advantage of absorbing mainly volatile non-polar compounds (www.sigmaaldrich.com). Hence, larger numbers of chemical compounds were recovered by using HS-SPME-GC-MS than from HS-GC-MS.

Among 41 chemical compounds identified by GC-MS, carbon dioxide was predominated in all kinds of bait. From previous reports, it had a strong attractiveness to many haematophagous insects, for example, the mosquito, *Anopheles gambiae* (Costantini et al. 2001). A combination of carbon dioxide with acetone and 1-octen-3-ol stimulates the behavioral response of the biting midge, *Culicoides impunctatus* (Bhasin et al. 2000). On the contrary, blow flies such as *L. cuprina* and *L. sericata* have no responses to carbon dioxide (Eisemann 1988, Wall and Warnes 1994). Hence, carbon dioxide could be excluded from the list of important attractants for *C. megacephala*.

Ammonia, a very volatile chemical, is known to attract several species of fruit flies and mosquitoes (Epsky et al. 1997, Robacker 1999, Smallegange et al. 2005). This gas contributes to cadaveric odor during the early decompositional stage of human corpse (Vass et al. 2004, 2008) and air over livestock farms (O'Neill and Phillips 1992). Cragg and Ramage (1945) revealed that the antennae of *L. caesar* and *L. sericata* are stimulated by ammonia, but their responses to this gas are not so strong in field studies (Cragg 1956). Also, ammonia is ranked the second most predominant

gas analyzed by both HS-GC-MS and HS-SPME-GC-MS. It could be assumed that ammonia can attract *C. megacephala*.

Indole is a common chemical detected from food for *C. megacephala* and its oviposition resources such as animal excrements; e.g., dog feces and horse dung (Johnson and Jurgens 2010), and decaying materials; e.g., pig carcasses (Dekeirsschieter et al. 2009) and human remains (Hoffman et al. 2009). As for blow fly olfactory-related attractant, indole combined with 3-methyl indole and ammonium carbonate yielded an effective bait for *Lucilia* spp. (Hobson 1937). Also, it acts as minor ingredient supplied in Lucilure and Swormlure (Urech et al. 1994, Hall 1995). In addition, the antenna of *C. hominivorax* elicits a response to indole as well as one to dimethyl disulfide (Cork 1994). In this study, indole was revealed from only high and medium attractive samples. Therefore, it could be accepted as an attractive candidate to *C. megacephala*. However, Aak et al. (2010) remarked that even though 90% of *C. vicina* flies take off when 1 mL of indole is introduced in wind tunnel, they do not move toward to the compound.

Small amounts of hydrocarbons were presented only from low and medium attractive bait for *C. megacephala*; 1-day tainted chub mackerel and fresh pork scrap, respectively. It is suggested that hydrocarbons; including dodecane, tridecane, pentadecane, pentadecene, tetramethyl pentadecane and methyl cyclohexene, are not important attractants for *C. megacephala*, as they are absent from any olfactory bait for blow flies, including larval wound fluid (Cork 1994) and rotten beef meat (Johnson and Jurgens 2010). Some of them (methyl propene, cyclohexyl cyclohexane and eicosane) are produced as minor components from pig carcasses (Dekeirsschieter et al. 2009); while nearly all; i.e., dodecane, tridecane, pentadecane and pentadecene,

are found in the air over raw lamb meat (Vasta et al. 2007). Regarding sex pheromone, long-chain hydrocarbons such as *cis*-9-tricosene (23 carbons) of *M. domestica* are related (Carlson et al. 1971). As for the sex pheromone of blow fly, this bait is ineffective for control management, since its volatility is low and only good for contact copulation (Carlson et al. (2007). Similarly, benzenoid compounds detected here; i.e., trimethyl benzene from 1-day tainted pork liver and ethyl benzenepentanoate from 1-day tainted chub mackerel, could be excluded from attractive candidate for *C. megacephala*, since their amount is too small for detection and no data on their blow fly attraction are available. Chinivasagam et al. (1998) suggested that trace aliphatic hydrocarbons and benzenoid compounds, which are detected from prawns, may be contaminated from environment or a natural constituent of its metabolism, since the abundances of hydrocarbons does not increased even when stored for more than 8 days.

All levels of attractive baits for *C. megacephala* produced various oxygenated compounds. Among these, acid compounds, which are rancid and sweaty smell, predominated in high and medium attractive samples. Small-chain acids comprising 2-6 carbons identified here are typical compounds previously reported from myiatic fluid (Cork 1994), decaying pig carcasses (Dekeirsschieter et al. 2009) and dog feces (Johnson and Jurgens 2010). Intensive study using EAG of Cork (1994) demonstrated that butanoic acid, pentanoic acid and 4-methyl pentanoic acid are attractive components to *C. hominivorax*. In addition, a combination of ethanoic acid, butanoic acid and pentanoic acid served as attractant to lure *A. rufifacies* and *Calliphora augur* (Ashworth and Wall 1994). However, the attractiveness of synthetic lure for *C. vicina* was reduced when adding butanoic acid and pentanoic acid (Aak et al. 2010). For the

long-chain fatty acids; octadecenoic and hexadecanoic acids, no information is available for any blow fly species, but they were found in larval frass and egg masses, and also served as oviposition attractive components for the Asian corn borer, *Ostrinia furnacalis* (Guo and Li 2009). Nevertheless, aromatic acids such as benzoic acid were included in the synthetic N-series attractant (Urech et al. 2004). On the other hand, benzoic acid relates (2-phenylethanoic acid and 3-phenylpropanoic acid), which were not found in this study, yielded low stimulation to the antenna of *C. hominivorax* (Cork 1994).

Other oxygenated compounds were presented in small amounts (<10%) and detection varied in samples; i.e., alcohols, aldehydes, esters and ketones. Three compounds; i.e., ethanol, hexanal and nonanal, are represented typically in fresh meat odor profiles (Larick et al. 1992, Ahn et al. 2000, Schindler et al. 2010) and butanone and heptanone have been reported in the air over pig carcasses (Dekeirsschietter et al. 2009). Hexanal, nonanal, heptanone can be detected together in several organs; i.e., muscle, fat and bone, over human remains. Otherwise, hexanal and nonanal are found mainly in blood, and the testes produce only heptanone (Hoffman et al. 2009). From the behavioral response test, propanol served as a low attractant to lure the fruit flies, *D. melanogaster* and *D. lativittata*, but it unattractive to *D. immigrans* (Hoffmann and Parsons 1984). Chadwick and Dethier (1948) demonstrated that *P. regina* avoids contact with various alcohols, aldehydes and ketones. For three esters, no report exists up to now on the presence of ethyl hexanoate, propyl pentanoate and ethyl octanoate, either as components of feeding and oviposition resources or attractive ingredients for any fly species. Besides, ester compounds such as methyl myristate and methyl palmitate derive from wasp nests and serve as repellents for the house fly,

M. domestica, and fresh fly, *Sarcophaga bullata* (Henderson et al. 1991). Furthermore, a phenolic compound characteristic of feces was released from 1-day tainted pork heart, intestine and muscle. Now, phenol can attract *C. megacephala*, since it serves as an ingredient in Swormlure (Hall 1995), and a combination of chemical bait consisting of phenol together with a variety of acids, alcohols and indole has more attractive potency in collecting *C. hominivorax* than traps baited with acids alone (Cork and Hall 2007). However, phenol has low or no impact on the electrophysiological and behavioral response of cattle flies (Birkett et al. 2004), and Shamsi et al. (1990) stated that phenol and phenolic compounds repel adult *A. rufifacies* and *L. cuprina*.

Various kinds of sulfur compounds were released from medium and high attractive baits; while, 1-day tainted chub mackerel gave low attraction to *C. megacephala* with no sulfur compound detected. Characterized by their rotten and decaying smell, the sulfur compounds detected here were methyl mercaptan, dimethyl disulfide, dimethyl trisulfide, dimethyl tetrasulfide and elemental sulfur. Of these, dimethyl disulfide and dimethyl trisulfide are well known as potential oviposition stimulant to myiasis-causing fly species and they are main ingredients in commercial attractants; i.e., Lucilure (Urech et al. 1994), Swormlure (Hall 1995) and Kiwitech bait (Morris 2005). Nilssen et al. (1996) stated that dimethyl trisulfide has strong attraction to various fly species in the families, Calliphoridae and Muscidae; while, two-combination of dimethyl sulfide and ethanethiol, and three-combination of dimethyl sulfide, dimethyl disulfide and dimethyl trisulfide attract *L. cuprina* and the carrion blow fly, respectively (Morris et al. 1998, Stensmyr et al. 2003). By using electrophysiological assay of antennal receptor neuron, the result revealed that

dimethyl disulfide stimulates the response of female *L. cuprina* (Park and Cork 1999). Furthermore, omission of dimethyl disulfide from the combination of Swormlure-4 causes a significant reduction in the number of *C. hominivorax* collected in field experiments (Green et al. 1993). Regarding these, sulfur compounds play a role in attracting the blow fly. Additionally, dimethyl disulfide might be the main attractive component that lures *C. megacephala*, based on several reasons: 1) it is used widely to attract the blow fly species, 2) it is produced from the feeding substrate of *C. megacephala* such as spoilage beef (Dainty 1996), pig carcass (Dekeirsschieter et al. 2009) and animal feces and rotten meat (Johnson and Jurgens 2010), and 3) a greater number of *Chrysomya* spp. (including *C. megacephala/saffranaea*) was collected in trap baited with formulation of dimethyl disulfide than in those with other sulfur formulations such as mercaptoethanol and sodium sulfide (Urech et al. 2004).

Even though amine compounds, such as trimethyl amine, putrecine and cadaverine, have been reported as a part of the attractive bait for synanthropic flies (Hwang et al. 1978, Yang et al. 1997) and decompositional volatile compounds from decaying pig carcasses (Dekeirsschieter et al. 2009), they were not detected in this study by HS-GC-MS or HS-SPME-GC-MS. This may be due to the different techniques used for volatile collection and GC-MS analyses between this study and previously published reports. For trimethyl amine, Chinivasagam et al. (1998) and Vasta et al. (2007) detected it in marine prawns and raw lamb meat by using dynamic headspace GC-MS (DH-HS-GC-MS), a technique that purges carrier gas through sample to increase headspace sample size. Triqui and Bouchriti (2003) used polar SPME fiber (divinyl benzene/carboxen/polydimethyl siloxane, DVB/CAR/PDMS) to collect trimethyl amine before GC-MS analysis of fish sauce. Putrecine and

cadaverine are analyzed generally by using high performance liquid chromatography (HPLC) (Wongyai et al. 1987), which has high potency for non- and semi-volatile chemicals. Amines are difficult to analyze by GC-MS, due to their low volatilities and interactions with the GC column, which leads to poor reproducibility. Before injection to GC-MS, Awan (2008) transformed non-volatile amine into volatile form by using the vaporization-derivatization technique. Furthermore, the sampling technique by using vaporization-derivatization method, together with polar SPME fiber (polydimethyl siloxane/divinyl benzene, PDMS/DVB), enhances the sensitivity of amines detection by GC-MS (Awan et al. 2008). However, amines such as trimethyl amine, putrecine and cadaverine, could be excluded from the attractive components for *C. megacephala*, since they lack of volatility and there is no information on attraction to the blow fly species.

According to GC-MS analyses, the results could reach the assumption that carbon dioxide and hydrocarbons (dodecane, tridecane, pentadecane, pentadecene, tetramethyl pentadecane and methyl cyclohexene) were excluded from attractive candidates of *C. megacephala*; while, ammonia, sulfur compounds (dimethyl disulfide, dimethyl trisulfide, dimethyl tetrasulfide and elemental sulfur), some short-chain acids (ethanoic acid, butanoic acid, pentanoic acid and methyl pentanoic acid), phenol and indole might be suspected as attractive components for luring *C. megacephala*.

Determination of suspected attractive compounds to *C. megacephala* was attempted by screening in the rearing cage and intensive testing with the wind tunnel. Only 5 commercial chemicals were tested by formulation of chemical-based baits, since some of them were limited in use by, for example, difficult handling (e.g.,

methyl mercaptan oxidized easily to dimethyl disulfide) or high price of the chemical product (e.g., dimethyl trisulfide and dimethyl tetrasulfide), which was not reasonable for further application. Of 41 chemical-based baits prepared (including preparations of single-use, chemical mixture, volatile mixture, aqueous dilution and paraffin oil-overlaying), responses of *C. megacephala* to 1, 3 and 5 mL of them were not higher than that for attractive fresh pork liver exudates, which act as a medium attractive producer. Firstly, it could be assumed that the concentration of ingredient might not be suitable for attracting *C. megacephala*. Ferry et al. (2007) used 20 μ L of dimethyl disulfide with paraffin oil dilution that varied from 0.1 to 100%, and results revealed that only 10% of the combination had potential attraction to staphylinids (genus *Aleochara*) and carabid beetles (genus *Bembidion*) in a field experiment. Secondly, *C. megacephala* may need some other chemical such as suspected phenol, indole and some acids that can be revealed by GC-MS analyses, but this has not been carried out. Cragg and Ramage (1945) noted that attraction of chemical compound is not effective when used alone, but a combination gives promising results. Thirdly, there could be differences in chemical properties of chemicals obtained from different suppliers. For example, dimethyl disulfide used in this study was purchased from Merck; while, dimethyl disulfide documented as attractive chemical to other blow fly species is obtained from Sigma-Aldrich (Park and Cork 1999, Urech et al. 2004, Cork and Hall 2007) and Fluka (Frederickx et al. 2011). One possible differences could be between isomeric structures of chemical, which impact on the attraction of chemical bait to insect response (McGovern and Beroza 1966, Warthen et al. 1994, Jang et al. 2001). Also, this study tested with ammonia solution; while, previous reports uses ammonium salt such as ammonium chloride (Mulla et al. 1977, Hwang et al. 1978).

To clarify the actual attractive chemicals for luring *C. megacephala*, more complexes of attractive formulations with precise ingredients, concentrations and ratios deserve for further attention. With polar SPME fiber such as PDMS/DVB, other polar chemical compounds, which might be attractive to *C. megacephala*, would be presented. For example, butanol has been found over pig carcasses (Hoffman et al. 2009) and it acts as a major ingredient in Swormlure (Hall 1995). Subsequently, adding internal standard and preparing in serial concentrations might be better obtaining a trend of concentration and ratio of attractive components.

Interestingly, the attraction of 1-day tainted pork scrap was extended by using traditional solvent extraction, similar to Robacker et al. (2000), who assumed that solvent media was able to dissolve attractants from attractive bait. The success in attractant extraction depends on a preferable interaction coefficient between extractant (= attractant in this study) and solvent (Maarse and Belz 1981). In this study, *C. megacephala* responded to attractant-extracted solutions more than their negative controls in the wind tunnel. With three extraction techniques, i.e., air-pumping, absorption and maceration, extracted solutions prepared by 24-hour maceration yielded the highest attractiveness to *C. megacephala*, followed by solutions incubated for 4-hour period of absorption and air-pumping technique, respectively. This indicated that incubation time and extraction technique impacted most on extraction. Direct contact extraction with 24-hour incubated maceration obtained more amounts of attractants than 4-hour absorption, and both of them yielded higher potentials for attracting *C. megacephala* than solution obtained by the air-pumping technique. Similarly, Robacker et al. (2000) revealed that adding water directly to duck feces for 24 hours incubation obtains a high attractive lure for fruit fly. Maarse and Belz (1981)

suggested that even headspace sampling, like air-pumping technique in this study, has the advantage of obtaining volatile compounds from food or other materials representing the actual odor, but it needs too much time to obtain a high concentration. In addition to the extraction technique and incubation time, kinds of solvent with difference in polarity also influence on the yield of extraction. As for the fruit fly, *A. suspense*, the attractants from attractive baits were extracted mostly in polar solvent such as water (Epsky et al. 1997). Carlson et al. (2000) found that attractive components for the stable flies, *Stomoxys calcitrans*, were more abundant in distilled water than other fractions of chloroform. Furthermore, acetone and hexane extracts have failed to lure the hard ticks, *Rhipicephatus pulchellus* (Zorloni et al. 2010). In this study, attractant-macerated water (AmDW solution) yielded the highest attraction among attractant-extracted solutions. Several explanations are possible as to why AmDW solution yielded higher attractiveness than other solvents, for example, 1) attractive components of 1-day tainted pork scrap could be dissolved well in water than other solvents. Cosse and Baker (1996) and Urech et al. (2004) used water as a solvent for producing blow fly synthetic lure, 2) adding water is an essential factor for microbe growth, and it might stimulate the spoilage mechanism (Adams and Moss 1995), 3) other solvents producing specific individual odor, change the characteristics of attractant-extracted solution, as in results of 50% and 70% ethanol, 4) other solvents might inhibit some mechanisms during the spoilage process such as ethanol and glycerol, which have the potential to inhibit growth of *Listeria monocytoges* (Oh and Marshall 1993), and some anti-oxidants of soybean oil could inhibit lipid oxidation (Tosic and Moore 1945), and 5) solvents may be able to collect attractive components, but have low ability in releasing them such as polyethylene glycol,

which acts as an anti-evaporant (Harburguer et al. 2011) or paraffin oil that has slow release (Birkett et al. 2004).

From GC-MS analyses, the volatile profile of AmDW solution comprised mainly components, as detected from 1-day tainted pork scrap and some additional compounds of propanoic acid, methyl propanoic acid, 2-methyl butanoic acid, methyl pentanoic acid, ethanol and nonanal. This indicated that additional compounds might reduce the attraction of AmDW solution to *C. megacephala*. Reduced attraction to *C. megacephala* is caused by reducing the concentration of AmDW, which indicates that *C. megacephala* responds to AmDW solution in a dose-dependent manner. Furthermore, changing the conditions of preparation such as limiting oxygen, salting with sodium chloride, adding phosphoric acid and sodium hydroxide, and pre-washed with boiled water and 70% ethanol suggested that *C. megacephala* seems attracted to specific ingredients with an actual concentration close to that of 1-day tainted pork scrap. This means conditions, with aerobe, low salt, neutral- and non-washing pre extraction, were as suitable for attractive component extraction from 1-day tainted pork scrap as that from AmDW solution. Even though sodium chloride has the advantage of producing more volatile chemicals (Maarse and Belz 1981), the main impacts were from inhibition that affects the feeding behavior of *P. regina* (Busse and Barth 1985), and microbe growth (Adams and Moss 1995). Differences in the relative abundance of acid against base might be expected to enable *C. megacephala* to distinguish between these attractive sources, as indicated from the attraction among neutral, acid and base preparations (AmDW, AmDW-AC and AmDW-BS, respectively). From the results of GC-MS, a larger amount of indole and sulfur compounds was yielded from base extraction; while, acid extraction obtained a

greater amount of acids and ethanol. However, it is remarkable that increasing short-chain acids, as in AmDW-AC solution, decreased the number of *C. megacephala* flies responding to bait when compared to AmDW and AmDW-BS solutions. The negative effect of additional acids is another explanation. Cork and Hall (2007) also revealed that more *C. hominivorax* flies were collected when the acid property of synthetic lure decreased. Likewise, the attractiveness of synthetic chemicals to *C. vicina* was reduced when adding butanoic acid and pentanoic acid (Aak et al. 2010).

AmDW solution overcomes long lasting attraction to *C. megacephala*, presumably water is essential for the fly to live. *C. megacephala* is found frequently to feed and oviposit eggs on a fresh corpse, which produces more moisture than an old one (Bohart and Gressitt 1951). Spradbery (1979) added some water to reactivate the attraction of tainted liver bait during his field trial. However, water had no attraction for *C. megacephala* in this study with experiments using distilled water as a control.

The attractions of baits investigated in the laboratory were similar to the assessments in the field, in that 1-day tainted pork scrap ranked as the most attractive bait to the wild fly species collected, followed by 1-day tainted beef scrap, AmDW solution and 1-day tainted chub mackerel, consecutively. Traps baited with 1-day tainted pork scrap and 1-day tainted beef scrap collected a greater variety of fly species over AmDW solution and 1-day tainted chub mackerel, especially at the forest site, which was defined as a high biological diversity area. In this study, the families, Calliphoridae, Muscidae and Sarcophagidae, revealed similar collecting patterns to those found with a sweeping net used in the same areas during rainy season of 2007, when the collection of flies in the family, Calliphoridae, were much larger than that of Muscidae and Sarcophagidae, consecutively (Moophayak et al. 2011).

C. megacephala and *A. rufifacies* were common species in these areas. Several blow fly species; *A. villeneuvei*, *C. chani*, *C. pinguis*, *H. tumrasvini*, *L. papuensis* and *L. porphyrina*, were distributed generally on high ground; while, *H. ligurriens* and *H. pulchra* predominated in the lowlands. Nevertheless, collection of *L. cuprina* was not recorded from these study sites. This clearly explains that *L. cuprina* is anautogenous flies, which complete its reproductive system without protein feeding (Spradbery 1979), and predominates near human dwelling such as market places.

Among the flies collected from the field experiments, adult *H. tumrasvini*, were collected at the forest site, where this fly species generally predominates (Kurahashi 1977). Information on the adult and larva of this blow fly species is currently very little. Kurahashi (1977) reported it as a new species from Thailand. In his description, the external male genitalia were illustrated. To fulfill the adult morphology, this study provides figures of the female ovipositors. In addition, color photographs of males and females were depicted to confirm the morphological identification. Similar to related blow flies such as *H. ligurriens*, the larval morphology of *H. tumrasvini* appears with 1) arrangements of prothoracic and mesothoracic spines, 2) a number of papillae on each anterior spiracle, and 3) the presence of inner projection on each posterior spiracle (Sukontason et al. 2007). These characteristics were supportive in part to the taxonomy given by Kurahashi and Chohanadisai (2001), in which *Hypopygiopsis* was placed in the same tribe as *Hemipyrellia*. However, they could be distinguished by using 2 criteria: 1) accessory sclerite; presence below the mouth hooks in *H. ligurriens*, but absence in *H. tumrasvini*, and 2) distance between posterior spiracle buttons; far apart (>0.2 mm)

in *H. ligurriens* (calculated from the scale bar in the figure of Sukontason et al. 2008) but close together (<0.2 mm) in *H. tumrasvini*.

In this study, large numbers of small muscids were trapped, with 19% of them identified as *Atherigona* spp., by using adult morphology and appearance of external male genitalia. Of all these small muscids, only *A. orientalis* had been recorded in Thailand (Pont and Magpayo 1995). In Brazil, this fly species has been reported as a mechanical carrier of helminthic eggs and larvae (de Oliveira et al. 2002). Also, research conducted in China found that *A. orientalis*, *Atherigona simplex* and *Atherigona* sp. were associated with animal carcasses, which suggested possible forensic importance (Shi et al. 2009). Pont and Magpayo (1995) stated that larvae of fly in subgenus *Acritochaeta*; including *A. orientalis*, act as scavengers or predators of decaying organic matter. Other flies in subgenus such as *A. maculigera*, *A. pallidipalpis* and *A. seticauda* have been reported as clear-wing flies from many countries around Asia; i.e., Nepal (Shinonaga and Singh 1994), India, Sri Lanka, Malaysia, Sumatra, Indonesia, the Philippines, Taiwan (Pont and Magpayo 1995). Otherwise, the wings of *A. setitarsus* were depicted with a dark narrow vertical patch along margin from midway between veins R2+3 and R4+5 to just beyond vein M, as reported from Vietnam (Shinonaga and Thinh 2000). Surprisingly, 4 specimens were not matched to any previous descriptions, as they formed unique features in not only the male terminalia, but also their general appearances, which were highlighted to 2 new species, namely *A. komi* and *A. thailandica*. *A. komi* presents two dark spots on apical of the wings and a distinct spiral groove on the dorsal aspect of the fore femurs; while, *A. thailandica* highlights a dark boomerang-shaped patch along the wing margin, extending from the distal $\frac{1}{3}$ between veins R1, R2+3 to just below vein M

and the fore femur, with two rows of long hairs on the dorsal surface. *Atherigona* (*Atherigona*) *soccata* was not collected in this study, which explains clearly that this fly species is attracted generally to sorghum (Sepsawadi et al. 1971, Pont and Magpayo 1995), and not decaying organic matter as in present baits.

The potency of AmDW solution among other baits yielded the greatest percentage of attraction to *C. megacephala*. In the laboratory investigation, a larger number of *C. megacephala* responded to AmDW solution than other fly species; *L. cuprina*, *A. rufifacies*, *M. domestica* and *P. dux*, which supports the finding of field experiment and suggests that AmDW solution could be a good choice for further application in aiming to control this fly species. Sex ratios of the *C. megacephala* collected were biased mostly toward females, as found in previous surveys that used protein-enriched material as bait (Ngoen-klan et al. 2011). Generally, protein products have the advantage of attracting gravid over non-gravid females, suggesting that protein products serve as an oviposition resource rather than a feeding substrate (Spradbery 1979, Tachibana and Numata 2006). Unlike laboratory investigations by Bunchu et al. (2008), the response of *C. megacephala* to 1-day tainted pork scrap was not dependent on sex, and in the results of this study, the mating status of *C. megacephala* had no impact on fly response to the bait. There are possible assumptions that cause different findings. Firstly, 1-day tainted pork scrap might serve as feeding attractant for *C. megacephala*. As for *C. hominivorax*, Hammack (1990) demonstrated that non-gravid females as well as gravid ones were attracted equally to feeding on sheep's blood and myiatic fluid. Secondly, females and some males were attracted directly to bait in general; while, mostly males stay near the bait. Thus, more females than males could be collected in the trap. Thirdly, the response of

C. megacephala to bait differs as its age changes, as in this study, when the number of *C. megacephala* responding to 1-day tainted pork scrap decreased when flies got older. This assumption is in accordance with the finding of Rathman et al. (1990) in that all ages of female flesh fly, *Sarcophaga bullata*, were attracted equally to protein bait; while, only young males fed on protein. Fourthly, it is possible that laboratorial flies responded to bait much more readily than wild ones, as occurs in *C. hominivorax* (Hammack 1991). Lastly, the reproductive status of *C. megacephala* may be mostly immature state during the rainy season, as examined in Malaysia by Mahon et al. (2004) who revealed that the juvenile blow fly, *Chrysomya bezziana*, predominated at 15-28 days post raining.

In addition to the previous discussion, this study provides valuable information on the environmental and physiological impact on response to bait from *C. megacephala*. In the experiment to optimize the most suitable time of day for the behavioral response of *C. megacephala* in the wind tunnel, the highest attraction was displayed in the afternoon (1-5 p.m.), which correlated with the best time to obtain the greatest number of these flies from the field (Sucharit et al. 1976, Sucharit and Tumrasvin 1981). The reason for this peak of fly activity in Thailand is still unknown; however, a combination of particular factors of the fly itself could be hypothesized, coupled with influential environmental factors; i.e., temperature and light intensity. For temperature impact, the fly, as a cold-blooded animal, is ready to express its complete behavior after accumulating enough heat. Flies that are tested in the morning (9-12 a.m.) are not hot enough because of the low nighttime temperature; whereas, those tested in the afternoon are warmed by the sun and are ready to express their complete behavior. Sunlight produces not only heat that warm the environment

during the day time, but also produces high light intensity, which could activate fly behavior, even though it is not a major impact. As for the fly itself, *C. megacephala* exhibits synanthropic behavior and may concentrate on searching activity during the afternoon, when more human activities occur. As a result of human activity, many organic waste materials are produced and decompose continuously, thus providing alluring odors that stimulate fly activity. A gradual increase in temperature and decreasing relative humidity in the natural environment also may enhance fly activity.

In this study, results on the response to temperature of *C. megacephala* in the rearing cage test agreed with the study by Ngoen-klan et al. (2011), in which a higher number of flies corresponded with higher temperatures, but not with higher relative humidity.

On the other hand, current results contrast to the finding of Sucharit et al. (1976), in which a negative correlation was found with temperature, but positive correlation with relative humidity. However, in all experiments with the wind tunnel, temperature and relative humidity were operated within a similar range, in both the morning and afternoon. Furthermore, to clarify the influence of either intrinsic or environmental factors on behavioral responses of flies, more experimental investigations are merited.

Since the amount of baits present can result in differences in the behavioral response of flies, it is vital to evaluate the quantity of bait used in experiments.

Gibson and Torr (1999) demonstrated that the number of cattle can increase the number of stable flies (*Stomoxys* spp.) attracted to hosts, but not for tsetse flies (*Glossina* spp.). In this current study, which used 100 g, 300 g or 1 kg of bait for attracting *C. megacephala*, no significant difference was caused in fly response in the wind tunnel at optimal wind speed (0.58 m/s). It was assumed that these amounts of bait had the same sufficient air-exposed area at the top of the bait mass to efficiently

release attractant odors, since they were held in a dish with the same diameter (14 cm). Thus, the larger mass of bait had no effect on response of the flies. This assumption is based on experimental results by Becher et al. (2010), which found by increasing the surface area of baits through sequentially reducing their size to a spray extract, a greater behavioral response was yielded in *D. melanogaster*, than when the original bait size was used.

To determine the most suitable number of flies for use in the wind tunnel, a behavioral investigation observed, and also recorded by video camera, that within 10 min after releases into the release compartment, flies moved toward the 1-day tainted pork scrap bait, regardless of whether there were 60 or 200 of them. However, as time passed, they moved both forward and backward to find landing sites in the trap compartments at either the baited or non-baited ends. When the 1-hour experimental period ended, flies in the group of 60 continued to land in the trap compartment at the baited end; whereas, flies in the group of 200 moved backward into the release and trap compartment of the non-baited end, thus yielding a lower attractive index in the latter group.

Wind speed is one of the primary factors in the wind tunnel for conveying odor and/or attractant molecules to reach sensory receptors of flies. Many experiments have shown that the use of an appropriate wind speed triggers more fly response (Cardé 1996, Gibson and Torr 1999). Absence of wind and low wind speeds result in only small, if any, behavioral responses from flies over long distances, and are primarily only appropriate over short distances (Marchand and McNeil 2000). However, too much wind speed may cause deformity of the odor structure and/or attractant molecules, such as odor plumes, and thereby reduce fly response (Cardé

1996). Geier et al. (1999) reported that the mosquito, *Aedes aegypti*, responds strongly to odor plumes of lactic acid, which is more filamentous in structure than homogenous. Experiments conducted with the Oriental fruit moth, *Grapholita molesta*, indicated that a decreasing amplitude of odor yields lower insect responses (Baker and Haynes 1989). Furthermore, excessive wind speed may affect insect flight directly, particularly in small or delicate species, as reported with the aphid parasitoid, *Aphidius nigripes* (Marchand and McNeil 2000), which may give the false impression of bait producing no attraction. On the other hand, wind speed also may change the overall behavior of insects. For example, high wind speeds (>2 m/s) cause the male potato aphid, *Macrosiphum euphorbiae*, to change its flying behavior to walking in response to female pheromones (Goldansaz and McNeil 2006). As for effects of wind speed on *C. megacephala*, the results in this study indicated that a wind speed of 0.58 m/s produces the most attractive index in a wind tunnel using 1-day tainted pork scrap as bait. This result was similar to the 0.50 m/s wind speed determined as optimal for stimulating and attracting this species (Bunchu et al. 2008) and another blow fly species, *L. sericata* (Wall and Fisher 2001).

Results revealed that a greater number of 5-10 day-old *C. megacephala* respond to 1-day tainted pork scrap when using a higher light intensity in the wind tunnel. This observation correlates with previous studies of *C. megacephala* (Bunchu et al. 2008) and *Calliphora vicina* (Aak et al. 2010). However, this finding is in contrast to other reports that state light intensity has only minimal effect on the behavioral response of diurnally active flies. Work published by Burkett and Butler (2005) demonstrated that light is not attractive to the diurnally-active mosquitoes, *A. aegypti* and *Aedes albopictus*; whereas, it was more attractive to *Culex nigripalpus*, a nocturnal species.

The attractive nature of light has been observed also in *Culex quinquefasciatus*, which has its active peak at night (Gowda and Vijavan 1993, Pipitgool et al. 1998). However, diurnally-active flies still require light for landing purposes (Burkett and Butler 2005). Absence of light or only minimal light intensity in experiments has been reported to cause lack of or minimal response to baits, based on the response to flowers of the diurnally-active walking thrips, *Frankliniella occidentalis*. In olfactometer and wind tunnel experiments, the absence of light yielded lower behavioral responses than those having light (Davidson et al. 2006).

Additionally, in experiments where bait was omitted from the stimulant compartments, about 36-60% of *C. megacephala* still moved from the release compartment into the trap compartments. This phenomenon has been observed also in other insects, which were being tested in choice assays (Cardé 1996, Costantini et al. 2001, Goldansaz and McNeil 2006). Clean air devoid of odor or attractant may stimulate olfactory receptor neurons in insects, thus causing flies to move toward the upwind stimulator (Vickers et al. 2001). Furthermore, other insects, such as cabbage root fly (Nottingham 1987), fruit fly (Budick and Dickinson 2006), and honeybee (Srinivasan et al. 1991), are more active to clean air when the wind speed was increased. However, this study was unable to state the interaction of the fly response between light intensity and wind speed because of no clear information on these factors. Budick and Dickinson (2006) noted that wind response of the fruit fly is determined together with visualization to high contrast objects; meanwhile, higher wind speed can inhibit the visual response. On the other hand, the visual response of insect can be influenced by both light intensity and wavelength (Nottingham 1987).