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In vivo real-time monitoring of aphrodisiac pheromone release of small white cabbage butterflies (*Pieris rapae*)

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ABSTRACT

The study of insect behavior is of practical importance for developing possible control methods in Integrated Pest Management. Currently, one model of butterfly mating behavior suggests that the initial location of potential mates occurs visually followed by the release of one or more short-range male aphrodisiac pheromones. This model is supported by data obtained from field observations and inferences based on the behavioral effects of chemicals extracted or isolated using indirect and offline techniques. In this study, we performed *in vivo* real-time monitoring of the male aphrodisiac pheromones released by the small white cabbage male butterfly (*Pieris rapae* Linnaeus) using confined direct analysis in real time (cDART) mass spectrometry. cDART is a new method easily adapted to the study in real time of chemicals released into the environment by virtually any insect. The major compound released by the male *Pieris rapae* was identified as ferrulactone. The experimental results reported here indicate that the release of ferrulactone occurs less than 1 s after the male visualizes its partner, and reaches a maximum after about one half minute. This study is the first reported *in vivo* detection and monitoring of butterfly male aphrodisiac pheromones in real time.

1. Introduction

Insects are well known to produce a diverse variety of sex pheromones to attract the opposite sex of the same species and to induce mating behavior (Karlson and Betenandt, 1959; Nieberding et al., 2008; Tillman et al., 1999; Witzgall et al., 2010). Characterization of these semiochemicals is important not only for understanding the biology and biochemistry of insects, but also for the environmental and agricultural sciences. Due to their diurnal lifestyle, butterflies have been considered as a microsmatic group of insects (Carlsson et al., 2013) that rely on visualization for communication and defense (Costanzo and Monteiro, 2007; Obara et al., 2008; Rutowski, 1991). Visual signals include color (Robertson and Monteiro, 2005) and ultraviolet reflectance (Obara and Hidaka, 1968; Papke et al., 2007), which may be complemented by chemical signals (Costanzo and Monteiro, 2007).

About 70 years ago, Tinbergen studied the courtship behavior of *Eumenis semele* Satyridae (Tinbergen et al., 1942). He suggested that mating was enhanced by the presence of some scent substances from the male butterflies. This insight was expanded by Brower and co-workers, who demonstrated that male *Danaus gilippus* use pheromones during courtship behavior to facilitate mating (Meinwald et al., 1966). Rutowski's studies have also shown that epicuticular components of male *Eurema lisa* and *Colias philodice* elicit acceptance behavior by females (Rutowski, 1977a,b, 1980). Subsequently, the utilization of pheromones by butterflies has been studied quite extensively (Hayashi et al., 1987; Kan and Hidaka, 1997; Karlson and Schneide, 1973; Nishida et al., 1996; Schulz and Nishida, 1996). The male aphrodisiac pheromones have been reported to be mainly released from secretory cells on the forewing in some species (Hedenstrom et al., 2015; Nieberding et al., 2008; Omura et al., 2001).

The small white cabbage butterfly (*Pieris rapae* Linnaeus) is common in many parts around the world. Despite wide geographic distribution and agricultural importance, their intraspecific chemical communication system has not been fully described. Recently, Yildizhan et al. (2009) have comprehensively studied the small white cabbage butterfly and the large white cabbage butterfly, *Pieris brassicae* Linnaeus. They found that the males of the two species possess two macrolide lactones on their forewings: ferrulactone ($C_{12}H_{18}O_2$) and brassicalactone ($C_{17}H_{26}O_2$), respectively. They further demonstrated that the two lactones enhanced mating success, suggesting that they are pheromones with aphrodisiac properties. Andersson et al. (2007) reported that a similar species, *Pieris napi* Linnaeus, releases some male specific pheromones

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comprised of geranial ($C_{10}H_{16}O$) and neral ($C_{10}H_{16}O$) in a 1:1 ratio. They suggested that the release of the above mixture is merely a passive physical process during flight.

So far, almost all chemicals released by insects for defensive, reproductive, and other purposes have been detected and identified using indirect and offline techniques, such as liquid extraction of whole body, wing, or organs (Lessman et al., 1989; Nishida et al., 1996; Schulz et al., 1993; Schulz and Nishida, 1996) or by ambient adsorption followed by instrumental analysis, usually with GC–MS. The advantage of these conventional approaches is that the volatile compounds can be concentrated in solvents or on the surface of the absorbing materials, leading to increased detection sensitivity. However, the methods need a certain period of time for sample collection, preparation and measurements (minutes to hours), thus they preclude any possibility of real-time analysis. The insect behavior and release of aphrodisiac pheromones are highly dynamic, and can quickly change with time and external environment. As the result, valuable information can be lost during the above sampling and analysis procedures.

The detection and characterization of semiochemicals that insects emit in real time should contribute significant new insights into their behavior. So far, very few studies have been reported in the literature that described the detection of volatile semiochemicals coincident with insect behavior in real time until the recent advancements of new mass spectrometry techniques. These new techniques allow one perform sampling under ambient conditions and observations in real time. The studies include the analysis of cuticular hydrocarbons of Drosophila (Yew et al., 2008), and female-female wasp behavioral contests (Goubault et al., 2006).

As a new atmospheric pressure ion source, DART has been successfully used in analyses of solid, liquid, and gaseous samples under ambient conditions without need of sample preparation (Chernetsova et al., 2011; Cody, 2009). Recent applications of DART include the analyses of organometallic compounds (Borges et al., 2009), medicinal plants (Kim et al., 2010), flavor and fragrances (Haefliger and Jeckelmann, 2007) and identification of volatiles from eucalyptus (Maleknia et al., 2009). A possible problem with gaseous sample analysis is the low sensitivity of the DART source due to open-air sampling. Recently, one of the authors developed the confined DART ion source (cDART), in which the plasma generated by the atmospheric pressure glow discharge ionizes gas-phase molecules in a T-shaped flow tube instead of open air (Li, 2012). The cDART design leads to an increase of ionization efficiency of gaseous samples by two or three orders of magnitude. The cDART source has been applied in real time analysis of plant wound response (Li, 2012) and online *in vivo* analysis of human breath (Li, 2013).

In this article, we report the experimental results of real-time monitoring of the aphrodisiac pheromones released by male small white cabbage butterflies (*P. rapae*). To the best of our knowledge, this report is the first *in vivo* real time detection and identification of pheromones released by butterflies coincident with their normal behavior.

2. Experimental section

2.1. Instruments

The experiments were performed with a Time-of-Flight mass spectrometer (AccuTOF JEOL USA Inc.), equipped with a DART ion source. The instrument was operated in the positive or negative ion mode with a resolving power of 6000 (FWHM). Mass spectra were acquired at a rate of one spectrum per second. Calibration for exact mass measurements was accomplished using polyethylene glycol (average molar mass 600) as the internal standard. Typical mass accuracy obtained in this study was less than 5 ppm. The exact mass measurements and isotopic distribution comparisons were used to determine the molecular formulas of the ions observed in mass spectra. In the experiments, the gas heater was set to 250 °C, the helium flow rate was 1 L/min, and the glow discharge needle potential and the grid voltage were set at 3.5 kV and 250 V, respectively. The AccuTOF MS settings are as follows: orifice 1 temperature = 80 °C, orifice 1 = 20 V, orifice 2 = 5 V, ring = 5 V. In the in-source collision-induced dissociation (CID) measurements, the orifice 1 voltage was increased to 65 V and 90 V, respectively.

2.2. Specimens

Most specimens of *P. rapae* were captured in the Silver Spring area of Maryland and identified by Dr. Brian Harris, Department of Entomology, National Museum of Natural History, Smithsonian Institution, Washington, D.C. Some specimens were raised privately and some were provided by Professor Nathan I. Morehouse of the University of Pittsburg.

2.3. cDART measurements

The experiments in this study were performed under the general room lighting (fluorescent light). The setup of the confined DART source is similar with the one used in the study of plant wound response (Li, 2012), as shown in Fig. 1. In brief, specimens of *P. rapae* were placed individually or together in a clear I-Chem jar (150 mL, 6 cm wide by 7 cm high, VWR LLC, PA) equipped with a lid containing a septum. Air was used as the carrier gas instead of nitrogen to prevent suffocation. In the experiments, a line of PEEK tubing (1/16″ OD, 0.010″ ID) from a compressed air cylinder was inserted into the septum and placed near the bottom of the jar. The second PEEK tubing (15 cm in length) was used to connect to the second jar or to the cDART ion source. The flow rate of air was controlled by a flow meter. Based on this setup, the gas molecules in the jars follow the air flow and enter the DART interface, where they collide and react with high energy metastable molecules produced by the atmospheric pressure glow discharge. The volatile compounds released by butterflies can be continuously monitored using mass spectrometry. Compared to the open-air DART source, the confined interface efficiently reduces random diffusion of the gaseous analytes in the air and can significantly increase collision reaction probability resulting in higher ionization efficiency. For the experimental setup used in this study, the strongest signal was obtained at the air flow rate of

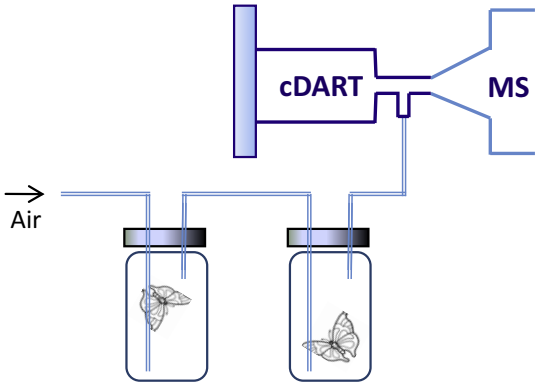

Fig. 1. Schematic diagram of the confined DART ion source used in *in vivo* real time monitoring of male aphrodisiac pheromone release of small white butterflies. One or two butterflies were placed in 150 mL jars. The specimen jars and cDART were connected using a 1/16″ (ID) peek tube. The air was used as the carrier gas at a flow rate of 0.2 L/min.