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A feeding trait study in head space of Silkworm *Bombyx mori* (Lepidoptera: Bombycidae) by GC-MS analysis

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Abstract
We analyzed the presence of chemical compounds from the head space of *Bombyx mori* which are attracted / recognized and entrapped by the head proteins upon biting of mulberry leaves through Gas Chromatography-Mass Spectrometry (GC-MS) analysis. The results of GC-MS showed that 28 chemical compounds were present in the head space of *B. mori*. Among this 28 compounds phenol, 2, 4-bis(1,1-dimethylethyl)-, benzene, 1,3-bis(1,1-dimethylethyl) and tetradecane are abundantly present, which indicate that these fours chemicals are the most important compounds present in the head of *B. mori* and responsible for feeding trait. The details of these compounds are discussed further.

Keywords: Silk worm, mulberry, GC-MS, *Bombyx mori*, chemical compounds

1. Introduction
The silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae) was domesticated from the ancient Chinese wild silkworm, B. *mandarina* Moore, and has experienced a history of artificial selection for more than 5,000 years [1]. The *Bombyx mori* is an oligophagous insect which feeds mainly on mulberry leaves due to its very long domestication.
India is the second largest producer of raw silk in the world after China with ranged from 15 to 18% of world production in last five years. Particularly, mulberry silk production was accounted more than 97% from the various state of india which includes (Andhra Pradesh, Jammu & Kashmir, Karnataka, Tamil Nadu and West Bengal) [2]. As these values have been increasing every year due to the largest consumer present in India, several studies have been conducted to increase the mulberry silk production, for example increase the growth of Mulberry Silkworm with biochemical compounds [3-5], development of disease resistant strain [6] and alteration of feeding behavior [7,8]. Today, due to water scarcity, energy supply, poor nutrition, emergence of new pest and viral diseases cause challenges in producing mulberry leaves, so there is a huge backslide in silk production by the formers throughout the world. The artificial diet also provides inferior growth rate of silkworm larvae compared to natural mulberry leaves [9, 10].
To overcome this problems, sericulture scientist are working for decades to find an alternative food for silkworm instead of mulberry leaves which are rich in high fiber content [11] and other nutritional values [12]. The terpenes found in the mulberry leaves, such as linalyl acetate, citral, linalool and terpinyl acetate as well as terpene alcohols attracts *Bombyx mori* larvae [13]. Silkworm attracted to herbivore induced volatiles offered a break in studying the molecular mechanism to trigger this behavior [14]. Preliminary research in silkworms that appear to express specific attraction towards mulberry in the larval antennae which are responsible for detecting the chemical queue and discriminating it, rather in adult antennae [14]. Moreover, chemical stimulants acting at a certain concentration at one receptor of the insect may influence acceptance of the host, while a different concentration of the chemical may cause the insect's central neuron to reject it [15]. In our study we analyzed the chemical compounds present in the head of *B. mori* with response to feeding behavior trait.

2. Materials and Methods

2.1 Experimental Animal

Disease free eggs of *B. mori* (the crossbreed race, Tamil Nadu White XNB4D2) were obtained from Government grainage center, Tiruchirappalli, Tamil Nadu, South India, and were maintained at a temperature of 27 ± 2 °C and a relative humidity of 75 ± 5% in the laboratory [16]. Hatched larvae fed with chopped tender leaves of the mulberry variety *Morus alba* (MR2) until third instar and with coarse leaves until the end of the last instar. Bed cleaning and spacing of larvae in trays were performed as described by Vanishree *et al.* [17]. The developmental stages were synchronized at each molt by collecting new larvae.

2.2 Sample Collection

The actively feeding 20 fifth instar larvae of *B. mori* were taken for this study. The surface of larvae was sterilized with povidone iodine and ethanol. The head was removed from the body with the help of a sterilized a scissor and it was kept in a 1.5 ml micro-centrifuge tube at -80º C for further process. The head was homogenized and separate by hexane fraction. The homogenized suspension was filtered and the filtrate was directly used for GC-MS analysis.

2.3 GC-MS analysis and data interpretation

GC analysis was carried out with PerkinElmer Clarus 500 Gas chromatography equipped with a Capillary Column Elite-5MS (5% Phenyl 95% dimethylpolysiloxane). The GC was equipped with a Column length of 30m and Column id 250µm. Using a temperature program of 50º C (1 min) at 8º C/ min to 150º C for 5min at 8º C/min to 280º C for 10 min. The homogenized suspension of the head space of *B. mori* was injected at 290º C using helium as the carrier gas (flow 1ml/min). Identification of compounds was based on the gas phase peaks obtained with mass charge values matching spectral library of GC-MS data system (NIST) library. The study was carried out from September - 2014 to May – 2015.

3. Results and Discussion

The larvae stage of *B. mori* is a voracious feeder at each molt from 1-5th instar. It is an oligophagous insect fed only *M. alba* leaf for its growth. Previously several report stated that c*is-jasmone* of *M. alba* plant was the major attractant compound for *B. mori* [18]. However this c*is-jasmone* is present in many plant leaves other than *M. alba*, but the insect have never prefer to feed other leaves. Many proteomic study are done in silkworm head showing a huge number of proteins identified revealing different properties form feeding behavior, among that 539 proteins were identified by shotgun strategy based proteome profiling by Li *et al.* [19]. In this aspect, chemical compounds from head space of *B. mori* will be much help full to gain information about feeding of mulberry leaves. So the present study was subjected to analyze the presence of chemical compounds from the head space of *B. mori* by using GC-MS analysis.

The head extracts of *B. mori* has small amount of relative low volatile compounds eluted before 30 min retention time by GC-MS analysis (Figure 1). The hexane extract of *B. mori* were observed 28 chemical compounds. Among them, 17 compounds belonging to hydrocarbons (61%) followed by 5 to alcohol compounds (18%), 3 to carboxylic acids (11%), each 1 ether (4%), ester (3%) and an aldehyde (3%) were observed in both males and females (Figure 2). Of 28 compounds, three major peaks were identified as phenol, 2, 4-bis(1,1-dimethylethyl)-, benzene, 1,3-bis(1,1-dimethylethyl)-, tetradecane which belongs to alcohol and hydrocarbon. The volatile constituents of head extract were tridecane (13.79%), benzene, 1,3-bis (1,1-demethylethyl (15.11%), tetradecae (18.44%), pentadecane (20.58%), phenol, 2,4-bis(1,1-dimethylethyl (21.26%), hexadecane (22.61%), eicosane (24.54%), 5-eicosene,(E) (26.37%), n-hexadecanoic acid (29.62%), tetratetracontane (33.96%) and sulfurous acid, 2-propyltridecyl (35.43%) were the major constituents of the total volatiles of the extract (Table 1). The compounds identified in the present study were compared with previous reports of volatile compounds present in the hexane extract of *M. alba*. Decane, tridecane, tetradecane, pentadecane, hexadecane and eicosane were previously reported which belongs to hydrocarbon group and present due to feed intake of mulberry *M. alba* [20]. Generally individual components of insects pheromones can also play different roles in different conditions [6]. Predominantly identified hydrocarbons in the present study has also been reported earlier and elucidated that hydrocarbons in epidermal cells (oenocytes) synthesizing and transporting hydrocarbons directly to the surface [21]. The function of hydrocarbons was not yet revealed very clear, some of the study showed the hydrocarbon may serve as solvent and as controlled release substrate for the more volatile aldehydes [22, 23]. The hydrocarbons were commonly found in insect cuticle which acts as pheromones. On the other hand, it is unlikely that all the compounds were also act as signal transmitters [24, 25]. The presence of alkane and alkene were the major compounds in the present study by MS analysis, it has also been reported that alkane compounds are familiar in head space with the frequency of 7 – 100% in mosquitoes *Culiseta longiareolata.* Surprisingly the predator of the *Culiseta longiareolata* named *Notonecta maculate* also persisted 12.5% frequency of alkanes in its head space responsible for the repulsion behavior of oviposition towards its predator [26]. These ultimately disclose that the predations of insects were also based on the hydrocarbons released by both predator and its prey. Numerous reports have stated that heteropterans produce defensive compounds in their scent glands against their predators. In stink bug *Dichelops melacanthus*, tridecane was a major synergistic compound which works along with other scent compounds as defensive [27]. Interestingly another group, [28] stated that the irritated stinkbugs *Tessaratoma papillosa* have the ability to release the volatiles which could create alarming effect to their congeners. Tridecane, tetradecane and pentadecane were the compounds identified in the stinkbugs in which tridecane was the predominant volatile which originates after irritation of the stinkbugs as an alarming compounds. The aldehydes and esters identified are strongly secreted, scented and irritants, providing both an easily detected warning signal and an effective defense. The compounds identified in this study will be used as ligands to go for a docking analysis with feeding specific olfactory receptors.

In this thereto, we validate the head compounds of *B. mori* similar with few compounds existing in *M. alba.* These head compounds can substantiate the attraction and repulsion behavior. Simultaneously there is an unresolved question raised about olfaction node, either it was due to plant volatiles or due to the head compounds. This evidence has brought out a new track of fact showing the head and plant compounds cophenetically responsible for the olfaction/chemosensing. Further deep analysis of exact binding reception compounds identification will pave a new way to find an alternative feed for Silkworm *B.mori*.

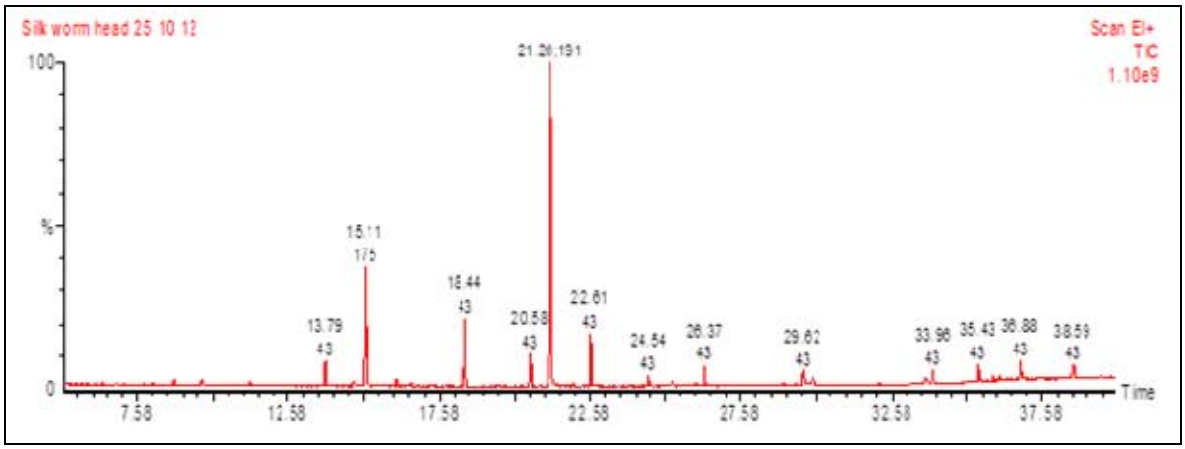

Fig 1: Gas chromatogram analysis of silkworm *B. mori* head space in hexane fraction

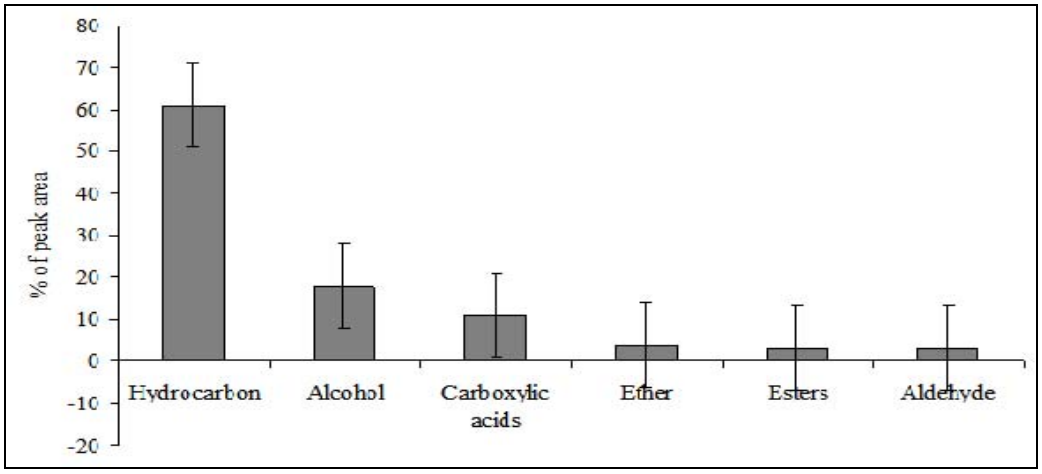

Fig 2: Major compounds in the hexane extraction from *B. mori* head space

Table 1: List of compounds identified from head space of *B.mori* by using GC-MS analysis

Compounds based on Groups	Hexane fraction	Formula	*M.alba* compound**
Alcohol	1,3-Hexanediol, 2-ethyl-	C8H18O2	
	Pentan-2-ol, 4-allyloxy-2-methyl-	C9H18O2	
	(S)-3-Ethyl-4-methylpentanol	C8H18O	
	Isotridecanol-	C13H28O	
	Phenol, 2,4-bis(1,1-dimethylethyl)-	C14H22O	
Aldehyde	Benzaldehyde, 2,4-dimethyl-	C9H10O	
Carboxylic acid	Tetradecanoic acid	C14H28O2	
	n-Hexadecanoic acid	C16H32O2	
	Octadecanoic acid	C18H36O2	
Hydrocarbon	Decane	C10H22	Decane
	Benzene, 1,3,5-trimethyl-	C9H12	
	1-Nonene, 4,6,8-trimethyl-	C12H24	
	Undecane, 5,7-dimethyl-	C13H28	
	Tridecane	C13H28	Tridecane
	Benzene, 1,3-bis(1,1-dimethylethyl)-	C14H22	
	Octane, 3,6-dimethyl-	C10H22	
	Tetradecane	C14H30	Tetradecane
	Pentadecane	C15H32	Pentadecane
	Pentadecane, 3-methyl-	C16H34	
	Hexadecane	C16H34	Hexadecane
	Eicosane	C20H42	Eicosane
	5-Eicosene, (E)-	C20H40	
	Hexadecane, 3-methyl-	C17H36	
	Nonadecane, 3-methyl-	C20H42	
	Hexatriacontane	C36H74	
	Tetratetracontane	C44H90	
Esters	Sulfurous acid, 2-propyl tridecyl ester	C16H34O3S	
Ether	Benzyl isopropenyl ether	C10H12O	

** Compounds already identified from *M.alba* leaf by GC-MS analysis [20].

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