EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 7
Issue: 3
47-52
Year: 2020

INSIGHTS OF SEMIOCHEMICAL COMPONENTS IN TERRESTRIAL LAND SNAILS

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Article Received on 02/01/2020 **Article Revised on 23/01/2020** **Article Accepted on 13/02/2020**

ABSTRACT
Semiochemical substances were detected for the first time in terrestrial land snail species (*Bradybaena similaris, Bradybaena pellucida & Bradybaena circulas*). We found snail behaviors resembling stimulation or attraction to these substances. These semiochemical substances act as attractant molecules or sex pheromones in snail species. No study has led to the isolation of semiochemical substances in land snail species. Here, we reported that land snails released semiochemical substances or sex pheromones for their communication.

KEYWORDS: Semiochemicals, Snails, Gas chromatography–mass spectrometry (GC- MS**)**, SPME.

INTRODUCTION

Scientific efforts for the discovery of chemical signal molecules have been going on for decades among living organisms. Mostly, published reports comprise insects and questions remain why not in other animals? Pheromones are sex pheromones released by an organism for sexual reproduction. It has been reported the possible role of pheromones in human sex and reproduction.[1] Identification of the sex pheromones of in the common forest looper *Pseudocoremia suavis* was described to study the developmental biology.[2] It was also showed for *Nasonia vitripennis*, a parasitic wasp with haplodiploid sex determination and female-biased sex ratios, that females use olfactory cues to discriminate against sperm-limited males.[3] Semiochemicals has been reported as sex-attracting substances in the marine insect *Trochopus plumbeus*.[4] *Prionus californicus*, a cerambycid beetle produce sex pheromone that attracts males identified by gas chromatography–mass spectrometry (GC-MS).[5] Determining the pheromone profiles could be useful to understand the mode of evolution of sex pheromones in particular species.[6]

Surprisingly, there are no chemical signal molecules/semiochemicals or pheromones described in snails or in broader term mollusks. Few reports described biological aspects of sex pheromones or other pheromones in snails. The apple snail *Pomacea canaliculata* was attracted by a water-borne sex pheromone with other individuals.[7] Studies on *Bradybaena similaris (BS)* and *Bradybaena pellucida (BP)* terrestrial land snail specimen described that either *BP* or *BS* must have sensed their differences chemically or physically during copulation. Also, their assortative mating suggests that they discriminate based on the differences in sexual pheromones.[8] A sex pheromone secreting gland has been discussed in terrestrial snail, *Euhadra peliomphala*.[9] None of the papers mention/indicate any signal substances or smeiochemicals in pulmonates.

In this paper, we demonstrated for the first time in the history of science that land snails use semiochemical substances for their communication. Some of these substances are might partially or fully part of a blend and work as attractant molecules in some terrestrial land snails in Japan.

Methods

Sample collection and storage

Adult and juvenile *Bradybaena similaris (BS)* specimen were collected from May to September, 2014 from Yamada Denki park, Matsumoto, Japan (36°12'55.1"N 137°58'17.4"E). Freshly collected adult snails were isolated and kept separated in plastic cases with moistened paper. The snails were given powdered cat food, malt and powdered egg shell in 4:4:2 ratios. Juvenile snails were treated in a same manner except they were kept together in a plastic case. Extensive care was taken to keep the snails in super healthy and active state. Snails were kept in climate control rooms when not used for experiments. *Bradybaena pellucida (BP)* specimens were collected from Chiba and Kanagawa prefectures (Japan), respectively. *BP* species were subjected to special care as they were very prone to body mite infection. *Bradybaena circulus (BC)* species were collected from Okinawa prefecture in Japan.

Air sampling/ Volatile extraction

Solid phase micro-extraction (SPME) was used as a technique to catch/extract volatile substances from active snails in laboratory conditions. We selected only super active snails prior to air sampling or volatile extraction. Precautions were taken appropriately to avoid unwanted volatiles during the extraction procedure. 100 µM PDMS (Polydimethylsiloxane) SPME fiber (SUPELCO, Japan) was utilized effectively for volatile extraction prior to volatile analysis. Other SPME fibers were also checked and ruled out in order to gain maximum peak and optimization. SPME fibers were manually used for the extraction procedure. SPME needle exposure time was set to 30 minutes after careful technical manipulations.

Volatile analysis

We used gas chromatography-mass spectrometry (GC-MS) for volatile analysis just after volatile extraction process. We utilized a GC (6890)-MSD (5973) system (Agilent technologies, Japan). SPME fiber was manually inserted in the GC-MS system for extracted compound analysis.

Bioassay

Bioassays were performed in two stages. We classified these stages as preliminary and final stages. In the preliminary stage, behaviour responses of the snails were carefully and thoroughly observed after placing the GC-MS detected compounds and an active snail in a glass container. We used different types, shapes and conditioned containers to rule out possible effect of other volatiles with air flow. In the final stage, we used Y-tubes for bioassay. The final stage experimental set-up is shown in Figure 5. We did many trials and repetitive observations. Statistical data were obtained using a Y-shaped glass tube bioassay.

RESULTS AND DISCUSSIONS

GC-MS analysis and volatile extraction

Freshly collected or maximum 2 months in captivity snails were used for volatile extraction by SPME. We investigated air-borne chemical substances among three snail species as shown in Figure 1. Initial experimental approach was done using *BS* specimen. Finding appropriate lab conditions for better semiochemical extraction was challenging and time consuming. It took quite a long time to find out proper containers for snail semiochemical extraction. Initially, besides observing snail behaviour in different types of containers,

Figure 1: Photo of *Bradybaena similaris (BS)* (a), *Bradybaena pellucida (BP)* (b), and *Bradybaena circulus (BC)* (c).

We compared behaviour of snails between hand-made mesh compartments and high quality glass vials. It was observed that the snails (*BS*) showed remarkbly high locomotory action and activity when placed in glass vials rather than metal mesh made compartments. It was also noticed that visualization of bevaiours, such as tentacle extensions, tentacles swingings, head-wart protrusion were prominent in glass vials.

Further, to be mentioned snails exhevitied frequent movement or extension of tentacles (both the large and small) in glass vials rather than in metal mesh compartments.

Placement of the glass vials in large glass container was also very easy. So, during the whole process of catching airborne semiochemicals (SPME) from snails, we used 20 cc glass vials with snails (isolated) placed in a large glass container. 30 minutes extraction time was chosen following better manipulation of experimental conditions. TICs (Total Ion Chromatograms) from adult and juveniles snail specimen were obtained and compared. Finding candiddate substance peaks were never so easy with so many background noise. In order to succeed, we followed several strategies, e.g., zooming and superimposing TICs, comparing peak retention times, visual detection of well-defined peaks, eliminating noise and false peaks etc. The first candidate substance, Carbamodithioic acid, diethyl-, methyl ester was detected in Adult *BS* species with good repeatation among GCMS runs. Figure 2 shows TICs of adult and juvenile *BS* species.

A well-defined peak at

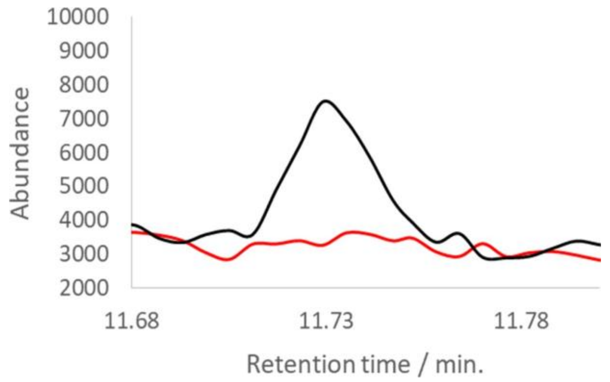

Figure 2: TICs from adult *BS* (black line) and juvenile *BS* (red line).

the retention time of 11.73 min. (approx.) can be clearly seen in case of adult *BS* species, whereas no peak at 11.73 min. (approx.) in case of juvenile *BS* species. This clearly suggests that adult *BS* individuals are releasing some semiochemical which is totally absent in juveniles. The peak at 11.735 min. (approx.) was analyzed and identified as Carbamodithioic acid, diethyl-, methyl ester ($C_6H_{13}NS_2$; MW: 163).

Sometimes, the peak appeared at the retention time of 11.729 min. (approx.) and was identified as 2-(Methylmercapto) benzimidazol ($C_8H_8N_2S$; MW: 164). First detected compound showed good repeatation among interday and intraday replicates. Interfering compound peaks were also found between retention times of 11.729 to 11.735 min. (Approx.). Some of the the interefering compounds identified by GC-MS were Isoproturon, Imipramine, Quinomethionate, and Pterin-6-carboxylic acid. We predict these compounds maybe from renal and faecal contents of the snails.

In case of *BP* species it was very difficult task to narrow down to possible candidate substance due to high number of peaks in TICs and more new compounds among replicates. We utilized several approaches to narrow down to candidate substances in *BP* species. One was the mucous test. We analyzed mucous volatiles. It was simple as we compared TICs of the control (tissue paper in 99.5% Ethanol) and TICs of the sample (snail used tissue paper in 99.5% Ethanol). We found 2-Myristynoyl-glycinamide and 9-Octadecenoic acid (Z)-, phenylmethyl ester which were also detected among replicates from SPME-GCMS tests. We even tried other different adsorbing material coated SPME fibers in search for common substances. Finally, after two seasons GC-MS tests with specimen data from two seasons specimen, we concluded to the following semiochemicals of the *BP* species: Carbamodithioic acid, diethyl-, methyl ester, 2-Myristynoyl-glycinamide, 9-Octadecenoic acid (Z)-, phenylmethyl ester, and Benzoic acid, 4-methyl, [4-(methoxycarbonyl)phenyl]methyl ester. We also extracted volatiles from *Bradybaena circulus (BC)* species.

Carbamodithioic acid, diethyl-, methyl ester, 2H-Pyran,2-(7- heptadecynyloxy)tetrahydro-, Benzene, (1-pentylheptyl)-, Ethyl geranyl acetate, Octadecane, 3-ethyl-5-(2-ethylbutyl)-, Butylated Hydroxytoluene were narrowed down based on repeatability, match criteria from GC-MS analysis software, nature/properties of compounds.

Bioassay with the detected compounds

At first, we closely monitored preliminary behavioural response of active adult BS snail species to the GC-MS detected compounds. Before that we had to go through analysing the nature/properties of the synthesized compound that was previously detected by GC-MS. We studied the nature of the candidate substances (Carbamodithioic acid, diethyl-, methyl ester and 2-(Methylmercapto)benzimidazol).

For example, solubility, volatility, and physical properties of the candidate substances were thoroughly analyzed. Carbamodithioic acid, diethyl-, methyl ester was found to have pungent odour and highly volatile. 2-(Methylmercapto)benzimidazol was found to be odourless, partially soluble in water and fully soluble in ethanol. Snail response to the candidate substances were observed and other effects, such as geotaxis and phototaxis were simultaneously observed and recorded. Initially, snail individuals were placed in glass containers with paper (Whatman filter paper) untreated with any compound (control) and on the other hand snail species were placed in glass containers with paper treated with candidate substances. At first, snails showed peculiar response. For example, when placed with the substance, Carbamodithioic acid, diethyl-, methyl ester, they tried to go to the compound source but suddenly diverted and showed unusual behaviour. But when the concentrations of the compounds were reduced the snails exhibited dramatic response. It was found that the snail species slowly crawled towards the paper with large tentacles pointed towards the paper treated with the compound. Even, some snail individuals touched the paper with their large tentacles in the vicinity of compound spots on the paper. We ruled out the phototaxis movement by carefully repeating the trials and minimizing light/shadow effect. Several types of snail activity/movement were carefully recorded. For example, we monitored the protrusion of the head-wart, active locomotory activity, approach to the compound source, and genital eversion/swollen. Figure 3 shows *BS* individual with genital eversion near the compound source during the preliminary observations of the treatments.

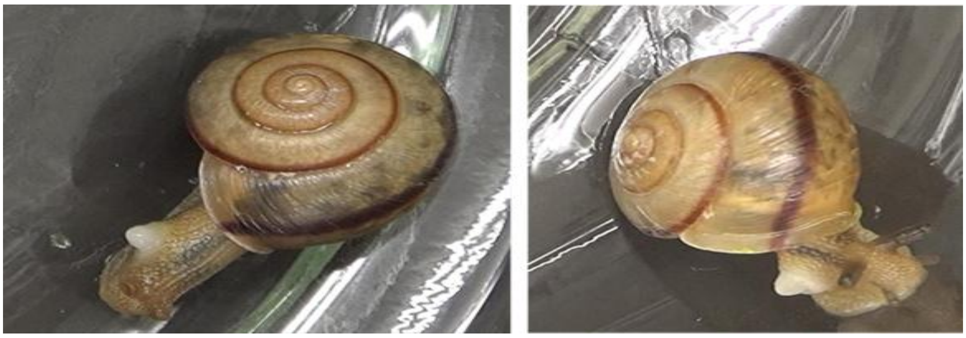

Figure 3: Response of BS adults to candidate substances during preliminary bioassay.

On the other hand, control snails showed poor locomotor activity, confused movement, no genital eversion, low tentacle movement etc. Furthermore, we found that the snail specimens were positively responding the candidate substance in much lower concentrations. Next, we also investigated snail response to another GC-MS detected compound [2-(Methylmercapto)benzimidazol)]. We found the *BS* snails were getting attracted at lower concentrations of the substances. As can be seen in Figure 4, we placed active adult *BS* snails in glass container with compound treated paper (small circled Whatman filter paper). The controls with compound untreated paper showed low locomotor activity of snails, usual movement, no approach towards the compound source etc. On the other hand, the treatment container showed the following activities:
High tentacle extension/activity, fast approach to the compound source, actively crawling and circling around the paper, touching the compound source with large tentacles, precopulatory behavior etc. Summary of the preliminary behavioral observation clearly indicates positive response to the candidate substances for the *BS* adult species.

Figure 4: Response of BS adults to candidate substances during preliminary bioassay.

We decided to perform bioassay with Y tube set up for testing the hypotheses from the preliminary findings. We completed the set up for the Y tube bioassay as shown in Figure 5.

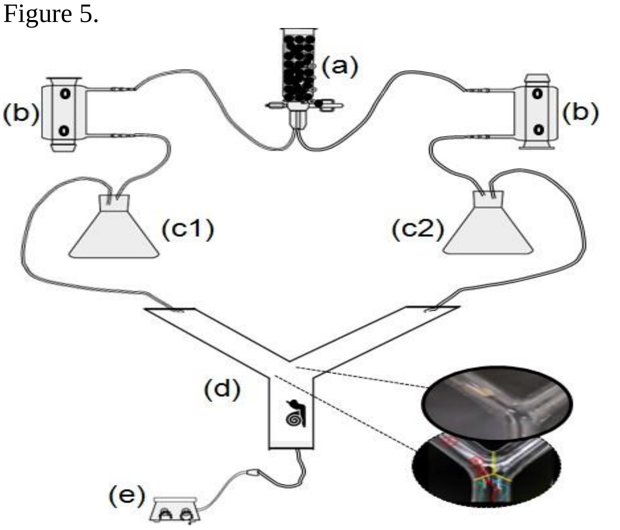

Figure 5: Y tube bioassay set up. (a) activated charcoal column, (b) flow meters, (c1, c2) glass containers served as control (without candidate substance) and treatments (with minute quantities of candidate substance, (d) glass Y tube, (e) mini air pump.

All the equipment, glassware, materials were carefully chosen, maintained and cleaned. As we can see in Figure 5, from the activated charcoal column to the Y tube and air flow meter, everything are connected through teflon tubes. Each time we performed or repeated the experiments new set of teflon tubes, and rubber stoppers of Y tube were selected to avoid contamination effect. The air-flow meter was used to adjust the pulling air and evaluated to see optimum /expected snail's actvity. An active snail was carefully placed in the Y tube as we can see in the illustration and the inset photo shows the snail's entrance towards the treatment arm of the Y-tube. One of the glass containers was kept empty or without the target compound and in the other the compound was placed in minute quantities. So, one served as 'treatment' and the other served as the 'control'. Snail entrance to the treatment arm of the glass Y tube is shown in the inset photo of Figure 5. Dramatic response of the adult snails to the compounds were observed in Y tube experiments as it was previously observed in preliminary bioassays.

We did series of bioassay experiments with active adult *BS, BP*, *BC* snails, young juveniles, and concluded as indicated in Table 1. We only selected the snails with active locomotory action and tentacle extensions for final stage bioassay tests. We named Carbamodithioic acid, diethyl-, methyl ester as compound A and, 2-(Methylmercapto)benzimidazol as compound B for easy mentioning. Results in Table 1, suggests that the *BS* adults are signifacntly choosing the compound source while Juvenile snails and *BC* adults are not. *BP* adults are also selecting compound A as the *BS* adults. This suggests compound A maybe acting as a common component of a blend or certain ratio of semiochemicals between the sister species. But, *BP* individials and *BC* individulas are not selecting compound B. Compound B maybe only acting for *BS* species.

Table 1: Final stage Y tube Bioassay results.

Test no.	Species	Compound	Compound Source	Control	Total	*P* (binomial test)
1	*BS*	A	13	0	13	<0.001
2	*BS young*	A	6	4	10	0.75
3	*BS*	A	8	0	8	<0.05
4	*BC*	A	2	6	8	0.28
5	*BS*	A	7	0	7	<0.05
6	*BS*	A	13	1	14	<0.05
7	*BS*	A	7	0	7	<0.05
8	*BS*	A	12	0	12	<0.001
9	*BP*	A	14	3	17	<0.05
10	*BS*	A	11	0	11	<0.05
11	*BS*	A	7	0	7	<0.05
12	*BS*	A	10	0	10	<0.05
13	*BS*	B	11	1	12	<0.05
14	*BS young*	B	2	5	7	0.45
15	*BP*	B	7	13	20	0.26
16	*BS*	B	14	1	15	<0.05
17	*BC*	B	4	8	12	0.38
18	*BS*	A	24	1	25	<0.001

CONCLUSIONS

Our study briefly opens first corridor to the world of semiochemicals in pulmonates, and renders a platform to continue further research to reveal molecular aspects of terrestrial land snails. It also gives ideas and insights to assist cutting edge research on signal molecules in molluscs. This study will help to build up chemical ecology research on mollusks.

ACKNOWLEDGEMENTS

This work was funded by the Grant-in-Aid for Scientific research from the Japan Society for the Promotion of Science (JSPS). We are grateful to Dr. Tomoko Okamoto and Dr. Shintaro Endo for their help and guidance. For field work and sample collection we are thankful to Kao Akiyama, Miki Takahashi, and Akari Kodera.

Disclosures: None.

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