Sasakia Charonda (Apaturinae: Nymphalidae) and its Three Related Butterfly Larvae Use Halitosis to Repel Predators

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Abstract

Accidentally, we discovered that *Sasakia charonda* (Nymphalidae: Apaturinae) larvae disturbed by ants or humans released volatile compounds from their mouth; thus, we tried to identify these halitosis. We collected halitosis directly from the mouths of *S. charonda* larvae into volatile-collecting tubes. Trapped halitosis were subjected to gas chromatography-mass spectrometry (GC–MS). We confirmed the identity of eleven substances by comparison to GC of known standards, and inferred them to be mainly alcohols and aldehydes/ketones, with main chains of 4–5 carbons. Three of the chemicals in these halitosis, 2-butanol, 1-penten-3-ol and 3-pentanone, affected the behavior of *Pristomyrmex punctatus* and *Formica japonica* ants that co-inhabited the *S. charonda* rearing cage. We concluded that the substances we identified in this study were used as defensive halitosis, analogous to osmeterium emissions specific to Papilionidae butterflies. Based on smell, Holikawa found that *Hestina assimilis* and *H. persimilis* larvae have closely related halitosis. Thus, we also analyzed the halitosis of these two species as well as *Apatura metis*, another Apaturinae, using the same methods. We found that these species also release halitosis. The composition of the substances of *H. assimilis* and *H. persimilis* were somewhat similar to that of *S. charonda*, whereas that of *A. metis* differed. Some of the substances also induced defensive behavior in these species of Apaturinae larvae.

Introduction

Many animals use specialized odorants to ward off predators. Among butterflies, Papilionidae larvae have a defensive tongue-like organ known as an osmeterium, which secretes a mixture of strong odorant substances that repel predators such as ants, wasps, and spiders. The osmeterial emissions by Japanese Papilionidae butterflies and their effects on two species of ants have been analyzed in detail (Honda 1983a), and information on osmeterial components has been compiled by Ômura et al. (2006).

Smedley et al. (2002) showed that larvae of the European *Pieris rapae* butterfly (Pieridae) also have defensive substances known as mayolenes, derived from 11-hydroxylinolenic acid and secreted from glandular hairs. In this study, the authors also revealed that mayolenes act as a potent deterrent against the ant *Crematogaster lineolata*, indicating a defensive role. Such defensive substances have not, however, been reported in other butterfly families.

As *Sasakia charonda* is the largest body-size species among Nymphalidae butterflies in Japan and neighboring countries, some researchers have a special interest in this species. *Sasakia charonda* has been bred since 2012 in the Kashihara City Museum of Insects, Kashihara, Nara by Taro Hayashi. While manually transferring *S. charonda* larvae between consumed and fresh *Celtis sinensis* (Rosales: Cannabaceae) leaves, Hayashi noticed that the larvae might have been emitting volatile compounds from their mouth. In response to handling, larvae raised their heads, opened their mandibles, and made clicking sounds, resembling “Kachi, Kachi” as if to threaten their predators. Moreover, some larvae made motions as if they were trying to bite the “predator”. Upon such stimulation, the larvae emitted halitosis. Moreover, Kaori Holikawa also found that same behavior was verified for *Hestina assimilis* and *H. persimilis*, as well

as Japanese Apaturinae species. Thus, we aimed to conduct a chemical analysis of the compounds released by these Apaturinae larvae. We also aimed to confirm that these compounds are deterrent to ants.

In addition, during this halitosis collection process, we noticed that the action of releasing halitosis behavior was transmitted to neighboring larvae. Therefore, we thought these halitosis must also act as alarm pheromone and examined whether these substances are effective against the Japanese emperor larvae.

Material And Methods

Collection of halitosis from Japanese Apaturinae larvae

The experiments were conducted at *Sasakia charonda* research center, Kashihara, Nara. *Sasakia charonda* larvae were reared in a 6.5 m × 5 m × 3 m (L × W × H) cage covered with a 1-mm nylon mesh net, and fed *C. sinensis* fresh leaves. We also used *S. charonda* larvae reared in the “Museum of Sasakia charonda in Hokuto, Yamanashi”. The sources, dates of origin, and additional information on the butterflies used in these experiments are shown in Table 1. Halitosis of 4th–6th instar larvae of *S. charonda*, *H. persimilis*, and *A. metis* were collected in sampling tubes (Tenax TA, GL Sciences, Japan) using an air suction pump with an initial suction capacity of approximately 20 L/min. To induce larval mouth opening, we pinched the posterior abdominal segments of the larvae with thumb and index finger. Upon this stimulation, larvae raised their heads and opened their mandibles, and the halitosis were collected (Fig 1). Because the amounts of each chemical were thought to be very small, we combined the halitosis expelled by 10 individual larvae of one species into each sampling tube. For collection of volatiles from *H. assimilis* larvae, a manual pump was used. The collection time for each larva was 15 s. Air just above the host-plant leaf surface was used as control. GC–MS analysis was performed on the samples at the Analyzing Center, Showa Denko Materials Techno Service.

Analysis of volatiles

The halitosis compounds were subjected to GC–MS analysis on an Agilent system consisting of a model 7890B gas chromatograph, a model 5977A mass-selective detector (EIMS, electron energy of 70 eV), and an Agilent ChemStation data system (Santa Clara, CA, USA). The GC column was a DB-VRX column (Agilent, USA) with 1.40 µm film thickness, 60 m length, and 0.25 mm internal diameter. The carrier gas was He, with a flow rate of 2.1 mL/min. The collected volatiles were analyzed using the thermal desorption method. The GC oven temperature program was 40 °C initial temperature for 3 min, followed by a temperature increase of 5 °C/min to 260 °C, which was held for 8 min. The mass-selective detector temperature was set to 230 °C. Sample components were identified by comparison of their mass spectral-fragmentation patterns against those stored in the MS library (NIST14 database) and by comparison to standards.

Reagents

As the results, we could detect 22 substances in the halitosis of *S. charonda* larvae. From these, we purchased 13 reagents, 2,3-butanedione (Wako, Fujifilm, purity > 98 %), 2-butanol (Wako, Fujifilm, purity > 99 %), 2-methyl-3-buten-2-ol (Tokyo Chemical Industry Co Ltd, purity > 97.0 %), 1-penten-3-ol (Wako, Fujifilm, purity = 95 %), 3-pentanol (Tokyo Chemical Industry Co Ltd, purity > 98 %), 3-pentanone (Wako, Fujifilm, purity = 98.0+%), 3-hydroxy-2-butanone (Tokyo Chemical Industry Co Ltd, purity > 98.0 %, may exist as crystalline dimer), 3-methyl-3-buten-1-ol (Tokyo Chemical Industry Co Ltd, purity > 98 %), (±)-2-methyl-1-butanol (Wako, Fujifilm, purity ?), 2,3-butanediol (Tokyo Chemical Industry Co Ltd, purity > 97.0 %), 3-methyl-2-butenal (Tokyo Chemical Industry Co Ltd, purity > 97.0 %), 3-hexen-1-ol (Wako, Fujifilm, purity ≥ 95 %), 2-methyl-2-pentenal (Tokyo Chemical Industry Co Ltd, purity > 97.0 %), and Cyclohexanol (Wako, Fujifilm, purity = 98 %), which we could obtain easily. These were used to confirm substances we could detect and behavioral testing of ants and larvae described below.

We also purchased Prenol (Tokyo Chemical Industry Co Ltd, purity > 98.0 %), Hexanal (Wako, Fujifilm, purity = 95.0+%), Benzaldehyde (Wako, Fujifilm, 98.0+%), 6-methyl-5-hepten-2-on (Wako, Fujifilm, purity = 96.0+%), Nonanal (Wako, Fujifilm, purity = 95.0+%), and Geranylacetone (*cis-trans* mixture, Tokyo Chemical Industry Co Ltd, purity > 96.0%GC) detected from other three species performed same examination.

Calculation of Retention Index of detected substances

We could not find the Retention Index (RI) for DB-RVX, so we calculated RIs of the substances we could detect. First, we analyzed mixture of Pentane, Heptane, and “analytical standard, contains C₈-C₂₀, ~40 mg/L each, in hexane, MERCK”, to know the retention times of those standard substances. Next, mixed reagents we could purchase, analyzed in the same manner as the halitosis samples, and the RI of these reagents were calculated. The RI of other substances were estimated from the RI of the substances before and after those.

For this calculation, we used the equation shown below.

$$I_A = 100 * n + 100 * (\log t_A - \log t_n) / (\log t_{n+1} - \log t_n)$$

Here, n are carbon number of *n*-H-(CH₂)_n-H, *t_n* are retention time of *n*-alkane, and *t_A* is the retention time of the substance that we want to calculate retention index those each retention time are located between those of *t_{n+1}* and *t_n*.

Effect of *S. charonda* halitosis on predator ants

This experiment was performed in the *Sasakia charonda* research center, Kashihara, Nara and Museum of *Sasakia charonda* in Hokuto, Yamanashi. Twelve confirmed substances were used to investigate their effects on *Pristomyrmex punctatus* and *Formica japonica* ants. We soaked a cotton swab with each substance and placed the swabs individually near the ants (Fig 2), and we observed whether the ants showed behavior to escape from the swabs soaked with the sample. The behavioral responses were recorded by a video camera (Standard resolution NTSC video, 720 × 480, HDR-HC7, SONY, Japan).

Effect of the *S. charonda* halitosis on the four Apaturinae species larvae

The substances identified in the rearing houses in Kashiwara and Hokuto were presented to *S. charonda* larvae using an olfactometer. One side of a silicone tube was connected to the outlet of an air pump, and the other side of the tube was connected to polyethylene pipettes. Small pieces of filter paper soaked in each volatile were inserted into each pipette as the source of substance (Fig. 3A). For the other three species, substance presentation was performed with swabs in the same manner as for the ants (Fig. 3B). We changed the substances by exchanging pipettes or swabs. We observed the behavior of the larvae in response to these substances.

Results

Identified substance and their retention index

The individual substances detected from the halitosis of the four species of larvae are summarized in Table 2. We were able to identify 13 of these volatiles: 2,3-butanedione, 2-butanol, 2-methyl-3-buten-2-ol, 1-penten-3-ol, 3-pentanol, 3-pentanone, 3-hydroxy-2-butanone, 3-methyl-3-buten-1-ol, ($\pm$)-2-methyl-1-butanol, 2,3-butanediol, 3-methyl-2-butenal, 3-hexen-1-ol, and Cyclohexanol. None of these volatiles were detected in the control air samples. The main chemical constituents of seven of the twelve substances identified consisted of a chain of 4–5 carbon atoms. Four of these substances had one methyl side chain. Seven were alcohols, one of which was a dihydric alcohol. One molecule, 3-Hydroxy-2-butanone, had both -OH and >C=O moieties. Another three had >C=O moiety, and one of them had two >C=O moieties. Three of the alcohols and one of the ketones were unsaturated. Only one cycloalcohol was confirmed.

We also analyzed the halitosis of the other three Apaturinae butterfly species in Japan. These results are also shown in Table 2. Among those, Prenol, Hexanal, Benzaldehyde, 6-methyl-5-hepten-2-on, Nonanal, and *trans*-Geranylacetone were confirmed using reagents.

The chemical composition of *H. assimilis* and *H. persimilis* halitosis samples slightly resembled that of *S. charonda*, although the halitosis of the two *Hestina* species mainly contained edulan-group substances instead of low molecule weight alcohols and ketones. In contrast, those of *A. metis* were different from those of the other three species, and mainly contained only 2-butanol and benzaldehyde, former was also found from other three examined Apaturinae larvae.

Calculated retention index of each substance were also shown in Table 2.

Effect of *S. charonda* halitosis on predator ants, and four Apaturinae species larvae

The results of responses of the halitosis to the ants are shown in Table 3. All of the examined ants responded in a relatively stable manner to 2-butanol, 1-penten-3-ol and 3-pentanone.

The responses of four results Apaturinae species larvae were also summarized in Table 3. Positive responses of the larvae to the substances were based on a specific behavior, the larva lifted its head from the host leaf and opened its mandible toward the pipette or swab. This behavior was only displayed by individuals who were resting on a scaffold formed by themselves during larval stage.

Discussion

In this study, we thought that we could show *S. charonda* and three other Apaturinae larvae use an halitosis to repel their predators. It is likely that the halitosis produced by *S. charonda* and the three other Apaturinae larvae have not been previously identified because breeders rear the larvae in the absence of predators. We could not collect substances from some individuals, because these larvae did not consider us a “threat” and did not open their mandible in response to our handling. It is well known (at least in Japan) that Papilionidae species larvae that are handled often by their breeders become accustomed to them, and, finally, they no longer extend their osmeterium in response to handling. It appears the larvae no longer consider the breeders a threat. Thus, we assumed that Apaturinae larvae also have this ability. We also noticed non-responsive individuals were in the process of walking or eating, at which time their aggression may be reduced.

The chemical composition of halitosis of *H. assimilis* and *H. persimilis* were similar to that of *S. charonda*; in contrast, that of *A. metis* was different. These differences may be because they utilize a different host plant. Another reason might be the phylogeny of these species; however, as the most recent phylogenetic study (Ohshima et al. 2010) on Apaturinae species did not examine *S. charonda*, we cannot draw a conclusion as to the relationship of the halitosis composition with phylogeny. We could conclude that, as the control samples did not contain these chemicals, they must be synthesized in the larval body *de novo*. The same has already been confirmed for the components of the osmaterium of the Papilionidae butterfly larva (Honda, 1983b).

We also noted the reaction spectra of halitosis of *H. persimilis* were very different from those of *H. assimilis*. Currently, *H. assimilis* and *H. persimilis* are thought to belong to same genus, *Hestina* Westwood [1850] (Type species: *Papilio assimilis* [Linnaeus, 1758]. Originally, *H. persimilis* was thought to belong to another genus, *Diagora* [Snellen, 1894], based on characteristics of its wing pattern. The differences in reaction between *H. assimilis* and *H. persimilis* to the halitosis might reflect this background. Alternatively, this result might be due to insufficient numbers of test individuals. Reactions of *A. metis* slightly resembled that of *S. charonda* and *H. assimilis*, contrary to expectations, because *A. metis* is thought to be phylogenetically different from the other three species. The constituents of halitosis of *A. metis* are also very different from those of the other three species; this may also be related to phylogeny or difference in host plants. However, the fact that the reaction spectra of *A. metis* resembled that of *S. charonda* and *H. assimilis* may be related to a shortage of examined individuals.

The defensive substances in the osmeterial emissions of Papilionidae species have been extensively studied (Ômura et al. 2006). According to this previous study, these substances consist mainly of

monoterpenoids, sesquiterpenoids, aliphatic acids, and their esters. Thus, the halitosis of the four Japanese Apaturinae larvae are very different from those of Papilionidae. As shown in Table 2, the defensive odor of the larvae of *S. charonda* and three related species is mainly derived from C₄–C₅ alcohol, which is also different from Heteroptera odors, which consist of aldehydes as described by Ishiwatari (1974). The synthesis pathways are unknown, and the mechanisms involved merit further investigation.

Odor-detecting systems of some ants have been researched morphologically and electrophysiologically (Hashimoto. 1992; Ozaki et al. 2005; Ruchty et al. 2008; Sharma et al. 2015), but might be not fully understood. Our results from ants were not consistent. We feel this relates to the fact that ants will judge individuals as probable nest mates by using very minute features of their cuticular hydrocarbon pheromones (Ruchty et al. 2008). However, among the chemicals in the larval halitosis, 2-butanol induced repellent behavior from almost all ants examined. As 2-butanol was the only substance detected in all four Japanese Apaturinae larval halitosis, this chemical must be the key repellent within the substance. Notably, these substances may also affect Apaturinae larvae by serving as alarm cues. Research on olfactory response of Lepidoptera larvae has been performed only with substances emitted from their host plants. Our results indicate that the larvae are also sensitive to substances which they produce and emit. Thus, our results may enrich the study of Lepidoptera olfaction.

Larvae of Apaturinae, Charaxinae, Boblinae, and Satyrinae species have a pair of horns on their heads, which they use defensively when attacked by a predator, as described by Teshirogi (2016). Nymphalinae, Heliconinae, and Limenitinae larvae have many spines on their body, and these may be used for physical defense from their predators. Moreover, Heliconius larvae have a chemical defense system, which is also found in Danainae and Ithomiinae larvae. In contrast, an effective chemical defense system is not known in Charaxinae, Boblinae, and Satyrinae larvae. In our current study, we could show the four Japanese Apaturinae larvae have a chemical defense system, and we wonder whether Apaturinae, Charaxinae, Boblinae, and Satyrinae larvae, found outside of Japan and whose larvae are morphologically similar to Japanese Apaturinae larvae, also bear such a chemical defense system. We hope other researchers will be interested in studying this issue.

In our study, we could not obtain sufficient larvae of *H. assimilis*, *H. persimilis*, or *A. metis* because a method of mass-breeding of these three species has not been established, such as that for *S. charonda*. We hope other researchers will tackle this vital research issue.

Declarations

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Tables

Table 1. Origin, dates and additional information of butterflies used

Sample	Origin	Date	Larval stage	Temp (°C)	<i>n</i>	Sampling duration (s)
<i>S. charonda</i> -0	Kashihara, Nara	2019 May 19	5-6	25	10	15
<i>S. charonda</i> -1	Kashihara, Nara	2019 May 19	5-6	25	10	15
<i>S. charonda</i> -2	Kashihara, Nara	2020 May 21	5-6	28	10	15
<i>S. charonda</i> -3	Kashihara, Nara	2020 May 21	5-6	28	10	15
<i>S. charonda</i> -4	Hokuto, Yamanashi	2020 Jun 11	4-6	20	10	15
<i>S. charonda</i> -5	Hokuto, Yamanashi	2020 Jun 11	4-6	20	10	15
<i>H. assimilis</i> -0	Aoba, Kanagawa	2020 Apr 16	4	20	3	30
<i>H. assimilis</i> -1	Tsurumi, Kanagawa	2020 Jun 29	5	27	3	60
<i>H. persimilis</i> -0	Aoba, Kanagawa	2020 Apr 16	5	20	5	30
<i>H. persimilis</i> -3	Setagaya, Tokyo	2020 Jul 02	5	27	1	30
<i>A. metis</i> -0	Minuma, Saitama	2020 Jul 02	5	27	4	60
<i>A. metis</i> -1	Minuma, Saitama	2020 Oct 20	5	22	2	30

Table 2. Volatile substances detected and identified from the halitosis of four Japanese Apaturinae species

RI	Found substances	Species	S. charonda	H. assimilis	H. persimilis	A. metis
511	2-methyl-3-buten-2-ol ^b		0			
591	2,3-butanedione ^a		0	0	0	
596	2-butanol ^a		0	0	0	0
600	Hexane ^a				0	
610	2-methyl-3-buten-2-ol ^a		0	0	0	
681	1-penten-3-ol ^a		0			
692	1-penten-3-on		0			
697	3-pentanol ^a		0			
700	3-pentanone ^a		0			
713	3-hydroxy-2-butanone ^a		0		0	
725	3-methyl-1-penten-3-ol		0			
733	3-methyl-3-buten-1-ol ^a		0			
737	2-methyl-1-butanol ^a		0			
773	3-methyl-2-buten-1-ol			0		
775	2,3-butanediol ^a		0		0	
782	Prenol ^a			0		
794	2,3-butanediol ^a		0			
804	3-methyl-2-butenal ^a		0	0	0	
806	Hexanal ^a				0	
832	4,5-dimethyl-2-hepten-3-ol		0			
841	3-hexen-1-ol ^b		0			
859	?				0	
889	?		0			
891	Cyclohexanol ^a		0			
984			0			

2-methyl-2-pentenal ^b				
993	Benzaldehyde ^a			0
997	6-methyl-5-hepten-2-on ^a		0	0
1116	?	0		
1123	Nonanal ^a		0	0
1294	Edulan-2 ?		0	0
1312	2-Undecane			0
1335	Dihydroedulan ?	0		
1336	Dihydroedulan ?		0	0
1342	Dihydroedulan ?		0	0
1350	Edulan-1 ?		0	0
1543	?		0	0
1559	trans-Geranilacetone ^a		0	
1796	?		0	

RI = retention index

0 = detected in that species

^a Confirmed by comparing to purified reagents

^b Not confirmed, but basic structures accurately estimated by comparing to purified reagents

The remaining are unconfirmed estimates because we could not purchase reagents of these.

Table 3. Response results to chemical samples

Examined substances	Ant species				Apaturinae species				
	<i>Pristomyrmex punctatus</i>		<i>Formica japonica</i>		<i>S. charonda</i>		<i>H. assimilis</i>	<i>H. persimilis</i>	<i>A. metis</i>
Location/Date	a	b	c	d	e	f	g	h	i
2,3-butanedione	o	-	O	O	5/5	10/10	2/3	0/1	2/2
2-butanol	-	O	O	o	5/0	10/10	0/3	0/1	0/2
Hexane	-	-	-	-	5/0	5/0	2/3	0/1	0/2
2-methyl-3-buten-2-ol	-	-	o	o	5/0	5/0	0/3	0/1	0/2
1-penten-3-ol	-	o	O	o	5/0	5/0	2/3	0/1	1/2
3-pentanol	-	-	O	-	5/5	5/0	3/3	0/1	0/2
3-pentanone	-	o	O	o	5/0	10/10	2/3	0/1	1/2
3-hydroxy-2-butanone ^a	-	-	O	-	5/0	5/0	0/3	1/1	1/2
3-methyl-3-buten-1-ol	-	-	O	o	5/5	5/0	2/3	0/1	0/2
2-methyl-1-butanol	-	-	O	o	5/5	10/10	3/3	0/1	1/2
2,3-butanediol ^b	-	-	o	-	5/0	5/0	0/3	0/1	0/2
3-methyl-2-butenal	-	o	O	-	5/5	10/10	2/3	0/1	1/2

On column a–d results from using ants, individual identification was impossible. O: Almost all examined individuals were responded, o: only some individuals were responded, -: no individual responded

a: Setagaya, Tokyo, 2020 Aug 01, n > 5. These ants were not co-inhabited any Apaturinae larvae.

b: Hokuto, Yamanashi, 2020 Aug 05, n > 5

c: Kashihara, Nara, 2020 Jul 27, n > 5

d: Hokuto, Yamanashi, 2020 Aug 05, n > 5,

On column e–i, A/B means, A = responded individual number, B = studied individual number.

e: Hokuto, Yamanashi, 2020 Jun 11/2020 Jun 24, last instar

f: Kashihara, Nara, 2020 Jul 26, 3rd instar

g: Tsurumi, Kanagawa, 2020 Jul 02/2020 Jul 03. last instar

h: Setagaya, Tokyo, 2020 Sep 13, last instar

i: Minuma, Saitama, 2020 Oct 20, last instar.

^a may exist as crystalline dimer

^b mixture of three isomers

Figures

Figure 1

Collection of halitosis from sixth -instar (final) larvae of *S. charonda*.

Figure 2

Ant (*Pristomyrmex punctatus*) responsive behavior to the volatiles. Setagaya, Tokyo, 2020 Aug 01

Figure 3

Non-responsive behavior to the substances. A: For *S. charonda* larvae, B: For larvae of *H. assimilis* and the other two species.

Figure 4

Positive response of *S. charonda* larvae to the examined volatile