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The chemical defenses of a stylocellid (Arachnida, Opiliones, Stylocellidae) from Sulawesi with comparisons to other Cyphophthalmi

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Abstract. Two female specimens of an undescribed species of stylocellid harvestman (Opiliones, Stylocellidae) from Sulawesi were extracted in methanol, and compounds in the product were identified by means of gas chromatography-mass spectrometry. Nineteen significant peaks were found, 12 of which were identified, indicating the presence of naphthoquinone, 2-tridecanone, 2-tetradecanone, 2-pentadecanone, 6-methyl-1,4-naphthoquinone, 6-methyl-1,4-naphthalenediol, and at least four unsaturated ketones. The spectrum differed both qualitatively and quantitatively from previously published data on *Siro exilis* Hoffman 1963 and *Cyphophthalmus duricorius* Joseph 1868 (Sironidae).

Keywords: *Siro*, *Cyphophthalmus*, exocrine products, ketones, naphthoquinones

Harvestmen (Opiliones) have a distinctive pair of prosomal exocrine glands opening to the surface via ozopores usually placed in the vicinity of the second leg pair. These glands produce a variety of substances (Table 1) effective in defense (e.g., Juberthie 1961a, 1961b, 1976; Eisner et al. 1971; Jones et al. 1977), and possibly with other functions as well. The secretions of approximately 48 species have been studied in detail up to this time. The results have been effectively reviewed by Gnaspini & Hara (2007; also see Hara et al. 2005), who presented a table listing the species and the compounds recorded from them. Forty-six compounds have been identified to date (numerous others are present but have not been identified), falling into the broad classes of long-chain alcohols and ketones, hydroxyquinones, phenols, and, more rarely, an alkaloid (nicotine), an amine (N,N-dimethyl-B-phenylethylamine), terpenoids (camphene, limonene) and bornyl esters (bornyl acetate and propionate) (Gnaspini & Hara 2007). A number of these compounds are unique or rare in nature, particularly in animals. Typically a single species produces a mixture of compounds, and the mixture of both identified and unidentified molecules may be characteristic of the particular taxon (family, genus, species) involved (Hara et al. 2005).

Although the defensive chemistry of Cyphophthalmi, Eupnoi and Laniatores has been studied for some species, that of many important higher taxa (i.e., Dyspnoi) remains completely uninvestigated. Our preliminary results (unpublished data) suggest that travunioids, only a single species of which has been examined so far, are extraordinarily diverse in their chemistry. Studies of Grassatores have concentrated on just a few families (Cosmetidae, Gonyleptidae, Manaosbiidae, Stygnommatidae) in this very diverse assemblage.

Defensive chemistry of the basal and divergent harvestman suborder Cyphophthalmi (e.g., see Giribet et al. 2002 for the phylogenetic placement of Cyphophthalmi) is interesting because if the composition of defensive secretions is of any phylogenetic use, its ancestral state in this group could be used to polarize the characters higher in the tree and to optimize the ancestral state of the defensive substances in Opiliones. But to date, only two cyphophthalmid species, *Siro exilis* Hoffman 1963 of North America, and *Cyphophthalmus duricorius* Joseph 1868 of Europe, have been studied for their defensive secretions (Raspotnig et al. 2005). Both of these species belong to the same family, Sironidae, while members of the other extant five families remain unexamined. The secretions of the two sironids consist of complex arrays of ketones and naphthoquinones.

In this paper, we present data on the secretion of an undescribed species probably belonging to the genus *Stylocellus*, family Stylocellidae. This species will be named and described in a forthcoming revision of the family by Ronald Clouse. In the current phylogenies of cyphophthalmids, the families Stylocellidae or Pettalidae appear basal within the suborder (Giribet & Boyer 2002; Boyer et al. 2007), although their stable placement is still debatable. The family Stylocellidae is found exclusively in Southeast Asia, from Southern China and Northeast India to the western side of New Guinea, in the Indonesian province of Irian Jaya, and the Philippine island of Palawan (Shear 1993; Clouse & Giribet 2007; Giribet et al. 2007; R. Clouse, in progress). The species we studied was collected on the Indonesian island of Sulawesi and is tentatively assigned to the genus *Stylocellus*, although the whole generic systematics of the family is currently under re-examination (R. Clouse & G. Giribet, in progress).

METHODS

Two female *Stylocellus* specimens of a yet undescribed species (Fig. 1) were collected alive in Bantimurung, Sulawesi (5°02′33″S, 119°44′08″E; 348 m elev.; Giribet locality number 512; collected 28 June 2008, R. Clouse, G. Giribet, C. Rahmadi, leg.; MCZ DNA101938), shipped alive to WAS, and extracted in about 0.5 ml of USP methanol. The extracts of the two specimens were pooled. The code number 06-172 was assigned to the specimens, both of which will be placed as vouchers in the Museum of Comparative Zoology, Cambridge, Massachusetts. The analysis of the extract was performed by THJ. Gas chromatography-mass spectrometry was carried out in the EI mode using a Shimadzu QP-5000 GC/MS equipped with an RTX-5, 30 m × 0.25-mm i.d. column. The instrument was programmed from 60° C to 250° C at 10°/min. Identification of components was accomplished

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Table 1.—Distribution of defensive secretions in Opiliones. For more detail, see Gnaspini & Hara (2007).

Taxon	Compounds
Suborder Cyphophthalmi	Ketones, naphthoquinones
Suborder Eupnoi	
Family Phalangiidae	naphthoquinones
Family Sclerosomatidae	ketones, alcohols
Suborder Dyspnoi	unknown
Suborder Laniatores	
Infraorder Insidiatores	Amines, bornyl esters, terpenes, alkaloids
	Phenols, methylquinones
Infraorder Grassatores	Benzoquinones, phenols

Figure 1.—One of the extracted specimens of stylocellid from Bantimurung, Sulawesi, photographed alive by G. Giribet. Specimen is about 6.5 mm in body length.

using NIST/EPA/NIH mass spectral library on CD-rom, version 1.7 (1999) and the NIST/EPA/NIH mass spectral library version 2.0d (2005).

RESULTS AND DISCUSSION

After gas chromatography-mass spectrometry examination, 19 significant peaks were identified in the extract. The major peaks in our analysis indicate the presence of naphthoquinone, 2-tridecanone, 2-tetradecanone, 2-pentadecanone, 6-methyl-1,4-naphthoquinone, 6-methyl-1,4-naphthalenediol, and at least four unsaturated ketones, two of which correspond to peaks S and U as noted by Raspotnig et al. (2005), so we have adopted their identification of these compounds (Table 2).

Table 3 compares the analyses of the three species of Cyphophthalmi. Chloronaphthoquinones are unique in the two sironid species (and as exocrine products of arthropods [Raspotnig et al. 2005]), but were not found in the stylocellid species; likewise undecan-2-one, 6-tridecen-2-one and 7-tridecen-2-one were absent from the secretion of *Stylocellus*. It is possible that these unusual compounds came from the males included in the sironid samples. Furthermore, the stylocellid secretion contained 6-methyl-1,4-naphthalenediol, a reduction product of 6-methyl-1,4-naphthoquinoine, which did not occur in the two sironids. The percent composition of compounds that were common to the three species shows strong differences between the stylocellid and the two sironids, and to a lesser degree, between *Siro exilis* and *Cyphophthalmus duricorius*. While 2-tridecanone made up about 20% of the secretion of both sironids, it comprised 50% in the stylocellid; 1,4-naphthoquinone and 6-methyl-1,4-naphthoquinone were found in very small amounts in the stylocellid, but at values from about 12% to nearly 18% in the sironids; pentadecan-2-one was at 13.8% for the stylocellid, but less than 1.7% for the sironids, and so on.

In summary, we may observe that while the composition of the secretions of the three species is similar in constituting mixtures of ketones and naphthoquinones, there are significant differences both qualitative and quantitative. Some of the differences could be due to the fact that we extracted our animals in methanol, while Raspotnig et al. (2005) used hexane. Raspotnig et al. (2005) found that the composition of the secretions in the two species studied by them was highly consistent from individual to individual within species, as has been reported in numerous previous studies of harvestman defensive secretions. This lessens our concern about the small size of our sample versus the much larger samples taken by Raspotnig et al. (2005); the remote location and relative rarity of the animals we studied makes it unlikely that large samples will be available in the near future.

The secretion of the stylocellid seems to contain fewer compounds than found by Raspotnig et al. (2005), who identified at least tentatively all of the 24 peaks they found (as opposed to our 19). However, very small peaks were not considered by us. Nevertheless, the observation about the

Table 2.—Compounds and percent composition identified in methanol extract of whole females of the stylocellid from Sulawesi. See also Fig. 2.

Peak	Compound	Relative %	Peak code in Raspotnig, et al. 2005
1	1,4-Naphthoquinone	2	E
2	2-Tridecanone	42	J
3	6-methylnaphthoquinone	1	L
4	Methyl branched 2-tridecanone	8	M or N
5	2-Tetradecanone	9	M or N
6	6-Methyl-1,4-naphthalenediol	1	Not detected
7	2-Pentadecadienone	8	S
8	2-Pentadecenone	7	U
9	Unsaturated ketone a	3	?
10	Unsaturated ketone b	3	?
11	2-Pentadecanone	14	W
12	2-Methoxy-1,4-naphthoquinone	3	Not detected

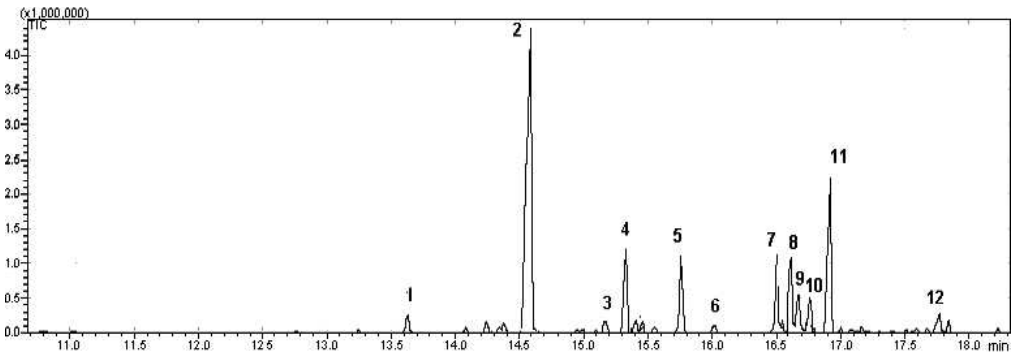

Figure 2.—Gas chromatographic profiles of whole-body methanol extract of stylocellid from Sulawesi. Identified peaks are numbered; numbers correspond to those in Table 2.

number of compounds would hold on the basis of the number of peaks alone. Aside from 5-methylnaphthoquinone, not found in the sironids, the stylocellid secretion is strongly dominated by ketones and lacks the diversity of naphthoquinones found in sironids. If stylocellids are in fact a sister group to other Cyphophthalmi, this observation suggests that the other naphthoquinones, especially the chlorinated ones, have been added in the course of evolution (or lost in the stylocellid lineage), and perhaps that ketones dominated the secretion of the common ancestor of extant Opiliones. Quinones may have been co-opted later from substances used by all arthropods to sclerotize cuticular proteins. However, proper polarization of this character requires first, a well-resolved cyphophthalmid phylogeny and second, a proper outgroup comparison. Since Opiliones' putative outgroups (Solifugae, Scorpiones, and Pseudoscorpiones; e.g., Wheeler & Hayashi 1998; Giribet et al. 2002; Shultz 2007) do not have this type of secretion, polarization may be difficult.

Raspotnig et al. (2005) pointed out that while both ketones and naphthoquinones are found in Cyphophthalmi, sclerosomatids (suborder Eupnoi) secrete only ketones (and alcohols) and the single phalangiid studied (also an Eupnoi), naphthoquinones. In Laniatores, Grassatores utilize phenols and methylquinones, while the single member of Insidiatores analyzed uses an eclectic mix of N.N-dimethyl-B-phenylethylamine, nicotine, bornyl esters, and terpenes. But again we must point out the strong bias in the data. Most of the species studied have been either Grassatores or sclerosomatids (see Table 1), while Dyspnoi and the Eupnoi family Caddidae, two groups of significant phylogenetic importance, have not been studied chemically at all.

In addition, no study has yet been made of the effect of collection method on the results of the analysis of harvestman secretions. In our studies, we are using methanol to extract whole bodies of live specimens, but other solvents, such as hexane, have been used in other laboratories. In some studies, live animals are induced to secrete their defensive compounds, which are collected either by micropipettes, fine glass tubing, or by absorption on filter paper. Thus we do not know if differences between species where different collection methods were used are real, or artifacts of the different methods. Certainly the fact that in many species the secretion of the repugnatorial glands is mixed with regurgitate from the gut could play a role if the already-mixed secretion is collected. The question of sample size also arises; here we used pooled extract from only two animals of the same sex, while

Table 3.—Comparison of percent composition of secretions of the stylocellid from Sulawesi with those of *Siro exilis* and *Cyphophthalmus duricorius* (data from Raspotnig et al. 2005). Confidence limits are given for the data on *S. exilis* and *C. duricorius* because Raspotnig et al. (2005) were reporting on individual extractions from 26 and 95 adult specimens respectively (for a complete list of compounds identified from the sironid species, see Raspotnig et al. [2005]). Our measurements are single points representing a pooled extraction of two adults due to the smaller collections of stylocellids when compared to the two sironid species studied previously. Bold figures represent the largest amount if significant differences are present; if two figures in a row are in bold type, there was no statistically significant difference between those two.

Compound	Stylocellid	*S. exilis*	*C. duricorius*
2-Tridecanone	**50.0**	20.28±3.79	20.21±3.56
7-Tridecen-2-one	not identified	15.47±1.35	**18.98±2.38**
1,4-Naphthoquinone	1.7	14.01±1.9	**17.61±2.82**
6-Methyl-1,4-naphthoquinone	1.0	**13.08±1.43**	**12.15±2.03**
Undecan-2-one	not identified	0.57±0.19	**9.71±1.59**
4-Chloro-1,2-naphthoquinone	not identified	**11.83±1.68**	7.09±2.44
6-Tridecen-2-one	not identified	**4.13±0.92**	**4.02±1.03**
Pentadecan-2-one	**13.8**	1.68±0.5	0.01±0.02
6-Methyl-4-chloro-1,2-naphthoquinone	not identified	4.30±1.24	0.36±0.51
2-Tetradecanone	**8.0**	1.10±0.33	0.65±0.25
Pentadecadieone	**8.0**	3.0±0.86	0.04±0.06
Pentadecenone	**6.7**	4.5±1.14	0.03±0.05
6-Methyl-1,4-naphthalenediol	**0.7**	not identified	not identified

Raspotnig et al (2005) used large samples including both sexes and juveniles, and analyzed each extract individually. Previous studies of harvestman defensive secretions vary greatly as to the numbers of individuals sampled, and in some cases numerous animals were used, but the samples were pooled for analysis. In the near future we hope to carry out a study using different collection methods and solvents on numerous individuals of the same species, in order to compare the effects of these methods.

Hara et al. (2005) were able to map the composition of the secretions of 22 gonyleptids (Laniatores, Grassatores) on a phylogenetic tree constructed from other characters. They found rampant homoplasy, but noted that closely related compounds (pairs of phenols and quinones) could easily be transformed into one another by oxidation or reduction, suggesting that ''families'' of such compounds could represent synapomorphies as transformation series of compound families. However, their final conclusions were ambiguous. On the one hand, it appeared that the great diversity of the secretions did not allow the recognition of phylogenetic lineages, with some exceptions, but on the other, they hoped that the analysis of more species and the addition of metabolic sequences and interchangeable compounds might provide phylogenetic information in the future. We agree with this position and plan to continue to analyze opilionid defensive secretions not only in a search for molecules new to science or new to arthropods, but also with the expectation that more data will help to clarify phylogenetic relationships within Opiliones.

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