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Frog Volatile Compounds: Application of *in vivo* SPME for the Characterization of the Odorous Secretions from Two Species of *Hypsiboas* Treefrogs

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Abstract A novel *in vivo* design was used in combination with solid-phase microextraction (SPME) and gas chromatography/mass spectrometry (GC/MS) to characterize the volatile compounds from the skin secretion of two species of tree frogs. Conventional SPME-GC/MS also was used for the analysis of volatiles present in skin samples and for the analysis of volatiles present in the diet and terraria. In total, 40 and 37 compounds were identified in the secretion of *Hypsiboas pulchellus* and *H. riojanus*, respectively, of which, 35 were common to both species. Aliphatic aldehydes, a low molecular weight alkadiene, an aromatic alcohol, and other aromatics, ketones, a methoxy pyrazine, sulfur containing compounds, and hemiterpenes are reported here for the first time in anurans. Most of the aliphatic compounds seem to be biosynthesized by the frogs following different metabolic pathways, whereas aromatics and monoterpenes are most likely sequestered from environmental sources. The characteristic smell of the secretion of *H. pulchellus* described by herpetologists as skunk-like or herbaceous is explained by a complex blend of different odoriferous components. The possible role of the volatiles found in *H. pulchellus* and *H. riojanus* is discussed in the context of previous hypotheses about the biological function of volatile secretions in frogs (*e.g.*, sex pheromones, defense secretions against predators, mosquito repellents).

Keywords Chemical defense · Glands · Skin · Smell · Solid phase micro extraction · Amphibia · Anura · Hylidae

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Introduction

There are comments and descriptions for over 100 species of frogs and toads that emit characteristic odors when stressed (Faivovich et al. 2013; Gallardo1961; Myers et al. 1991; Smith et al. 2004b). Some of the volatiles perceived by humans are described as curry, earth, grass, mercaptane-like, mink-like, nut, onion, thyme, and vanilla. Similar organoleptic patterns may be shared by species that are only distantly related, whereas phylogenetically closely related species may produce drastically different odors (Smith et al. 2004b). These observations raise the question about the origin of volatile secretions in anurans (*de novo* synthesis or sequestration), about their biological significance, and their possible use for taxonomic purposes (Faivovich et al. 2013; Smith et al. 2003, 2004a).

Despite the high number of anuran species releasing odorous volatiles, only a few studies have pursued their

identification, and only six publications dealt with this issue (Poth et al. 2012, 2013; Smith et al. 2000, 2003, 2004b; Starnberger et al. 2013). One reason for this deficiency may be difficulties inherent in the process of sampling volatiles through solvent extractions, which is a critical step in the analytical procedure (*e.g.*, Myers et al. 1991). A successful approach that has helped to overcome this limitation is solid-phase microextraction (SPME; Arthur and Pawliszyn 1990). SPME integrates sampling, extraction, pre-concentration, and sample introduction into a single solvent-free step, as analytes in the sample are extracted directly and concentrated on the extraction fiber (Vas and Vékey 2004). Headspace SPME (HS-SPME) in combination with an effective separation technique (GC), and a selective and sensitive analytical method (mass spectrometry; MS), has become the most widely used procedure for the analysis of volatile organic compounds (VOCs; Stashenko and Martínez 2007; Vas and Vékey 2004).

In vivo sampling using SPME is highly attractive in studies that involve living organisms because it is non-invasive causing minimal disturbance to the investigated system (Musteata and Pawliszyn 2007). In fact, applications, such as consecutive analysis of the same individual at different times, would not be feasible with any other method because target organisms would seriously suffer or even be killed (Ouyang et al. 2011). Two studies have proved the usefulness of SPME to determine the odors of frogs through *in vivo* sampling (Smith et al. 2000, 2003).

So far, only few volatiles have been identified as emissions of anurans: monoterpenes from *Litoria ewingi* (Smith et al. 2000), monoterpenes, β-caryophyllene, and a lactam (2-pyrrolidone) from *Litoria caerulea* (Smith et al. 2003, 2004a), alkanols and lactones (low molecular weight macrolides and mantidactolides) from three species of mantellid frogs (Poth et al. 2012, 2013), and mixtures of sesquiterpenes, fatty acid esters, alcohols, and macrolides, from 11 species of hyperoliid frogs (Starnberger et al. 2013). β-Caryophyllene is sequestered from environmental sources in *L. caerulea* (Smith et al. 2004a), which poses the question of the origin of other monoterpenes in frogs, whereas other compounds seem to be biosynthesized *de novo*. The function of these compounds is unknown in most cases. The only exceptions are two macrolides from *Mantydactylus multiplicatus* that were previously shown to act as sexual pheromones (Poth et al. 2012). However, Starnberger et al. (2013) proposed that sesquiterpenes, alcohols, and macrolides also may play a role in sexual communication of hyperoliids. Terpenes from the secretions of *L. caerulea* were associated with defensive strategies against predators and mosquitos (Smith et al. 2003; Williams et al. 2006). The paucity of data found in the literature, demonstrates the necessity to identify volatiles occurring in other species of anurans, which will increase the knowledge on their chemical nature, origin, and potential biological role.

Hypsiboas pulchellus and *Hypsiboas riojanus* are two common frog species that belong to the family Hylidae, which, like several other species of *Hypsiboas* and related genera, release a strong odor when handled (Barrio 1962, 1965; Faivovich et al. 2013; Gallardo 1958, 1961). In particular, in *H. pulchellus* the odor has been described as crushed plants (Faivovich et al. 2013), or skunk- or fox-like odors (Gallardo 1958, 1961; Langone '1994' [1995]). The present study aimed: (1) to characterize the volatile components present in the skin secretions of both species under a simulated stress situation by using *in vivo* HS-SPME; (2) to determine whether the source of the compounds is located in the integument by comparing results from *in vivo* HS-SPME with HS-SPME from isolated skin samples; (3) to trace the origin of components by comparing blends of volatiles released by newly caught specimens with those from specimens kept under lab-controlled conditions for 15 months; (4) to examine whether the frogs have an uptake system, sequester and accumulate volatile compounds from environmental sources into the skin, considering the volatiles occurring in the terraria in which the frogs were kept and in the insects used to feed them; and (5) to discuss the possible function of the identified compounds in terms of proposed functions for odoriferous secretions of amphibians.

Methods and Materials

Animals

Specimens of *H. pulchellus* were collected at two different localities (BL and TA). BL, Brazo Largo, province of Entre Rios, Argentina (33°40′41″S, 58°52′56″W), and TA, Estação Ecológica do Taim, state of Rio Grande do Sul, Brazil (32°44′32″S, 52°38′02″W). Both localities were more than 400 km apart. Four and 14 specimens, collected in August 2013 from TA and BL, respectively, were sampled using optimized extraction conditions. Additionally, specimens from Osorio state of Rio Grande do Sul, Brazil (29°54′42″S, 50°17′51″W) and some specimens from TA had been collected in April 2013 and were used to optimize extraction conditions. Specimens of *H. riojanus* were collected from Laguna el Rodeo (LR), province of Jujuy, Argentina (24°06′23″S, 65°28′46″W), in August 2013. In all cases, individuals were transported to the laboratory and kept in plastic terraria between 6 and 10 day before being sampled. During this time, they were provided with dechlorinated water and kept under 14:10 hr L:D cycle conditions at room temperature (16–28 °C). It is worth mentioning that during field and laboratory handling, the characteristic smells of both species were perceived in both sexes when the individuals were incidentally stressed. Considering these findings, male specimens were preferred, because they occur more frequently and are easier

to capture in the field. Using only males was also a way of standardizing the samples. Nevertheless, two female specimens of *H. pulchellus* from BL that had been kept under laboratory conditions over 15 months were analyzed. All remaining samples were obtained from male specimens. Species determination was carried out according to Cei (1980), with taxonomic changes introduced by Faivovich et al. (2004, 2005) and Köhler et al. (2010).

In order to determine whether the compounds are *de novo* biosynthesized by the frogs or originate from the diet, eight specimens of *H. pulchellus* and four specimens of *H. riojanus* were collected in BL and LR, respectively. These individuals were kept under similar laboratory-controlled conditions as described, but for a period of 15 months. During this time, they were fed on a diet of mealworms (*Tenebrio molitor*) and crickets (*Achaeta domestica*), obtained from a commercial supplier. For comparisons, we will discriminate the conditions and breeding period of the specimens within the text, as NAT condition, or simply NAT (for those specimens collected in the field and analyzed within 10 d after capture) or LAB condition (LAB, for those specimens collected in the field and held under lab-controlled conditions for 15 mo prior to analysis). Specimens that were killed for skin sampling are stored as voucher specimens in the herpetological collections of the Museo Argentino de Ciencias Naturales "Bernardino Rivadavia"- CONICET, and the Universidade Federal do Rio Grande do Sul (UFRGS). This study was carried out according to the regulations specified by the Institutional Animal Care and Use Committee of the Facultad de Ciencias Exactas y Naturales, UBA (Res C/D 140/00).

Reagents and Materials

Extractions were performed using the following SPME fiber coatings: divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS; 50/30 μm of thickness), carboxen/polydimethylsiloxane (CAR/PDMS; 85 μm), polyacrilate (PA; 85 μm), polydimethylsiloxane/divinylbenzene (PDMS/DVB; 65 μm). All fibers were obtained from SUPELCO (Bellefonte, PA, USA). Commercial standards benzene, benzonitrile, ethylbenzene, 1-ethylmethylbenzene, eucalyptol, 1-decanol, 1-hexanol, 3-hexanone, isoprene, limonene, 3-methylbutanal, 3-methyl-1-butanol, 2-methyl-3-pentanone, 2-methyl-2-propanol, 1-pentanol, 2-pentanone, 3-pentanone, 2-phenylethanol, styrene, toluene and *p*-xylene were purchased from Sigma-Aldrich (St. Louis, MO, USA), whereas 4-methyl-1,3-pentadiene was purchased from ChemSampCo (Chicago, IL, USA). Linear alkanes of the series C8–C30 used for the determination of linear temperature programmed retention indices (LTPRI), were purchased from Sigma-Aldrich. Additionally, linear alkanes of the series C5–C7 were isolated in the laboratory from available solvents obtained from Nuclear (São Paulo, SP, Brazil). For the *in vivo* sampling, a 50 ml clear glass cylinder, open at both ends, was employed, and specifically modified for this study (Fig. 1). The bottom side of the cylinder was covered with a glass lid, whereas the top was covered with a conical glass accessory (five cm in length, and open at both ends). The outside of the cone had a small opening that was covered with a septum, through which an SPME fiber was inserted. Additionally, the topside of the cylinder had glass ridges to prevent the frog from escaping and two small lateral holes (2 mm) that were covered with septa obtained from SUPELCO. In this way, the entire device (hereafter, *in vivo* setup; Fig. 1) was hermetically closed. Sampling of volatiles from isolated skin samples of frogs as well as dietary analyses were performed in 22 ml SPME vials (SUPELCO).

SPME Collection from *In Vivo* Samples

Live frogs were sampled using *in vivo* static-headspace solid phase microextraction (*in vivo* S-HS-SPME) according to the

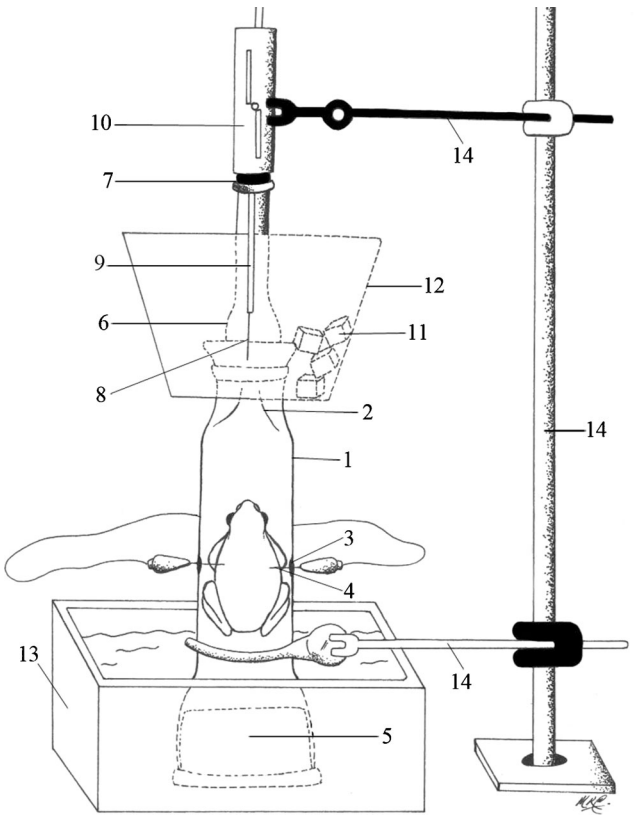

Fig. 1 Schematic diagram of the device used for *in vivo* sampling of volatile secretions from the integument of frogs by SPME. Numbers indicate: 1, 50 ml glass cylinder (14.3 cm long and 3.5 cm diam; 2, glass ridges; 3, septum covering a lateral glass hole in the cylinder; 4, platinum electrodes on the dorsum of a frog; 5, glass lid covering the bottom side of the cylinder; 6, conical glass accessory covering the top side of the cylinder; 7, needle hub and septum; 8, coated fiber; 9, needle; 10, commercial SPME holder; 11, ice cubes; 12, Styrofoam cup; 13 water bath at 35 °C; 14, metal supports. The electricity source connected to the electrodes is not shown

following procedure. The individuum was carefully removed from its terrarium and was freed from urine by applying a gentle pressure to the bladder. It then was placed inside the *in vivo* setup from its bottom side, which was immediately closed with the glass lid, whereas the conical accessory was used to cover its topside. The fiber was exposed once the frog remained motionless (approximately 1 min). Subsequently, two platinum electrodes were inserted through the small holes at the lateral sides of the cylinder. These electrodes allowed the application of a gentle electrical stimulation of 2–3 V with 2 msec of pulse duration (Tyler et al. 1992) on the dorsal region of the frog for 10–20 sec to trigger discharge of glands. The stimuli were repeated ten times at regular intervals during the extraction procedure. After sampling, the fiber was immediately inserted into the injection port of the gas chromatograph. Five min after being released from the device, the frog exhibited a normal behavior. Because of the individual variation in the size of *H. pulchellus*, either one or two individuals were used in a single sampling procedure in the same glass cylinder. By this way, the total skin surface stimulated was approximately the same for each experiment.

Before each experiment, the *in vivo* setup was washed with a non-ionic detergent and was heated to dryness. After cleaning, a blank trial of this device was performed using the same extraction conditions (*i.e.*, time, temperature), but without the frog. The fiber was desorbed into the injector port of the GC/MS instrument using the same experimental conditions as for the frog samples. This blank run was termed *in vivo* blank setup, and allowed to trace volatile contaminants that might have influenced the results of the subsequent test. We also performed two different analyses with the same individual in order to examine whether the frogs secreted volatile compounds as a natural process, or due to the application of the electrical stimuli. Both experiments were run under similar conditions, but whereas in the first analysis the individual was left undisturbed within the cylinder during the whole sampling procedure, in the second run, the individual was electrically stimulated. These tests were performed only with some of the specimens [*i.e.*, NAT specimens of *H. pulchellus* (two from TA and six from BL) one LAB specimen of *H. pulchellus* from BL, and two NAT specimens of *H. riojanus*].

Optimization of Collection Conditions for the *In Vivo* S-HS-SPME Procedure

Various tests were performed to find the best extraction conditions. These tests were run exclusively with *H. pulchellus*. First, we evaluated the efficiency of the fibers DVB/CAR/PDMS, PDMS/DVB, CAR/PDMS, and PA. Second, we tested two different temperatures: room temperature and a thermal gradient (Fig. 1). The thermal gradient was generated as follows: the bottom side of the cylinder was disposed vertically in a water bath at 35 °C, whereas the topside was cooled down to 5–8 °C. The low temperature was achieved through ice cubes that were disposed around the external side of the conical accessory within a Styrofoam cup. Finally, four extraction times were compared (5, 10, 20, and 40 min). According to the results of these tests, the best conditions for *in vivo* S-HS-SPME with *H. pulchellus* were a DVB/CAR/PDMS fiber and a thermal gradient. A collection time of 20 min gave reasonable signal-to-noise ratios and represented a relatively low level of stress to the frogs. Consequently, running times of the experiments were set to 20 min.

SPME Analyses from Isolated Skin Samples

Although the glands located in the integument are the most plausible source of volatiles from these species, Gallardo (1958) had suggested that they were emitted from glands located in the mouth. Therefore, in order to determine whether they were effectively secreted from the integument, volatiles from isolated skin samples were analyzed by using HS-SPME. The procedure was as follows: the same individuals that were sampled using *in vivo* S-HS-SPME were freeze-killed and stored at −20 °C for 2–4 days. Ten min prior to use, two specimens from identical tests [species, locality (only for *H. pulchellus*), and specimen condition] were removed from the freezer and left at room temperature until thawed. Subsequently, the skin from the dorsum and flanks was dissected from each specimen, placed in empty glass vials of 22 ml, and submitted to ultrasound for 10 min. The vial was placed in a water bath at 55 °C, and skins were sampled using the S-HS-SPME procedure by exposing a DVB/CAR/PDMS fiber for 40 min. Following sampling, the fibers were immediately used for gas chromatographic analyses. In order to trace contaminants from the vial, a blank run was performed before placing the skins. This blank trial was carried out under conditions identical to those used during the extraction of frog skin samples. For comparisons, analyses performed with living frogs and with isolated skin samples will be referred hereafter, as *in vivo* sampling or skin sampling.

Analysis of Food and Environment as Possible Sources of Volatiles

Sampling of volatiles from the diet was undertaken with crickets and mealworms that were used to feed the frogs under LAB condition. Groups of three medium size crickets and mealworms were freeze-killed at −20 °C. Ten min prior to use, they were removed from the freezer and were left at room temperature until thawed. Subsequently, the insects were placed in an empty glass vial of 22 ml, and some small cuts were performed by a scissor. The vial was closed and submitted to ultrasound for 10 min. The headspace was sampled under the same conditions as described for the skin sampling.

For terraria sampling, a DVB/CAR/PDMS fiber was exposed for 1 hr at room temperature (22–24 °C) under three different conditions: (1) terrarium with two frogs resting on its side, with the fiber placed at 5 cm from the specimens; (2) the same terrarium 5 min after removing the frogs; (3) the same terrarium without the frogs after having been cleaned with a non-ionic detergent and air dried. Dietary and terrarium sampling were performed in triplicate.

Chromatographic Analysis

GC/MS analyses were undertaken on two similar instruments from the Universidade Federal do Rio Grande do Sul (UFRGS) and Universidade Federal de Santa Catarina (UFSC). These instruments were Shimadzu (Kyoto, Japan) GCs coupled to Mass Spectrometer Detectors (GC/MS QP 2010 Plus). The system from UFRGS was equipped with a Restek Rtx® -5MS column (60 m×0.25 mm×0.25 μm) obtained from Restek (Bellefonte, PA, USA), whereas the system from UFSC was equipped with a Restek Rtx® -5MS column (30 m×0.25 mm×0.25 μm). All analyses performed for the optimization of extraction conditions (excepting the PA fiber; UFRGS equipment) were done with the UFSC equipment, whereas all analysis performed using optimized extraction conditions (including terrarium and dietary sampling) were carried out with the UFRGS equipment. In both systems, the carrier gas was helium (purity of 99.9999 %; White Martins, RS, Brazil) with a flow rate of 1 $ml.min^{-1}$. The oven temperature was programmed as follows: initial temperature of 40 °C (3 min hold), 7 $°C.min^{-1}$ to 230 °C, 50 $°C.min^{-1}$ to a final temperature of 230 °C (3 min hold). The injector was operated in splitless mode, and its temperature was held constant at 250 °C. The fiber desorption time was 10 min. A narrow bore (0.75-mm I.D.) liner was employed in order to achieve sharp SPME injection bands. A quadrupole mass spectrum detector was operated in the electron impact mode (EI) at 70 eV and 240 °C, and the mass range 40–400 u. All samples, including linear alkanes and commercial standards, were run under the same chromatographic conditions.

Data analyses were performed using the Shimadzu GCMS solution software Version 2.53 SU3, which includes the NIST21 and NIST107 Mass Spectral Libraries. Compounds were tentatively identified comparing experimentally acquired mass spectra with mass spectra reported in commercial NIST mass spectra libraries, and by analyzing their EI fragmentation patterns. Also, experimental LTPRI were compared to data reported in the literature from the same (or equivalent) column. Peaks with signal-to-noise ratio (S/N)≥3 and a minimum of 92 % of mass spectrum similarity were considered as detected and tentatively identified. Peaks that presented S/N <3, but met the mass spectrum similarity mentioned and had similar retention data to those from peaks with S/N >3, were also considered as tentatively identified but critically evaluated. For instance, when differences between species were only observed at this signal-to-noise ratio, they were not considered as species-specific compounds. Unambiguous identifications were assigned when standard compounds were available.

Results

S-HS-SPME-GC/MS Analysis of Frog Samples

In total, 42 volatile compounds were identified in all samples analyzed from *H. pulchellus* and *H. riojanus* (Table 1). The compounds formed complex mixtures, and the chemical structures could be grouped according to nine different criteria: aromatic hydrocarbons, aliphatic and aromatic alcohols, aldehydes and ketones (carbonyl compounds), alkenes, esters, sulphur containing compounds, and nitrogen containing compounds. Aliphatic alcohols formed the most abundant group (15 compounds, 35.7 %), followed by carbonyl compounds (eight compounds, 19.0 %), whereas four compounds (9.5 %) were grouped separately as monoterpenes. Figure 2 shows overlaid total ion chromatograms (TIC) obtained from the individual (with and without stimulation) of *H. pulchellus* from BL (LAB specimen) using *in vivo* sampling. Figure 3 shows the TIC corresponding to the analysis of skin samples from NAT specimens of *H. riojanus*. In both figures, peak numbers are the same as compound names listed in Table 1, which are numbered according to their retention times (t_R). These numbers are indicated in bold within the text.

Visual inspection of data (Table 1) showed differences in the number of compounds identified after *in vivo* and skin sampling, which suggests that the procedure employed influenced the analytical results. A detailed examination allowed recognizing that 12 compounds were present only in skin sampling (**10**, **13**, **22**, **24**, **30**, **33**, **38**, **39**, **42**, **40**, **3**, **37**), whereas six compounds (**4**, **14**, **8**, **17**, **28**, **35**) were present only in *in vivo* sampling. Of the 12 compounds that occurred in skin samplings, nine were aliphatic alcohols, whereas those that occurred in *in vivo* samplings showed other structural features. Differences were not significant between species and among specimen conditions (NAT or LAB), within each species. However, a detailed inspection showed that compounds **17**, and **34** occurred exclusively in *H. riojanus*, whereas **18**, **22**, **19**, **31**, and **37** were found only in samples from *H. pulchellus*. With respect to specimen condition, the observed differences were due mainly to monoterpenes, which occurred only in NAT specimens from both species. These results will be analyzed in detail considering each of the nine different

Table 1 Volatile compounds identified in *Hypsiboas pulchellus* and *Hypsiboas riojanus*

Peak n°	Identity	LTPRI exp.	LTPRI lit.[a]	Samples[b, c]									
				H. pulchellus						*H. riojanus*			
				in vivo sampling			Skin sampling			*in vivo* sampling		Skin sampling	
				TA NAT *n*=2	BL NAT *n*=3	BL LAB *n*=6	TA NAT *n*=1	BL NAT *n*=2	BL LAB *n*=2	LR NAT *n*=4	LR LAB *n*=2	LR NAT *n*=2	LR LAB *n*=1
Aliphatic alcohols													
2	2-Methyl-2-propanol[d]	524	526[A]	1	1	6	–	–	1(1)	3	2(1)	2(1)	1(1)
4	2-Methyl-3-buten-2-ol	612	614[B]	2	1	6(1)	–	–	–	3	2(2)	–	–
6	2-Methyl-2-butanol	639	634[C]	–	1	–	–	2(2)	1	2	2(2)	2(2)	1(1)
10	1-Penten-3-ol	682	682[D]	–	–	–	1(1)	2	1(1)	–	–	2	1
12	3-Methyl-1-butanol[d]	734	734[E]	2	–	6(1)	1	2	2	–	–	2	1
13	2-Methyl-1-butanol	738	738[B]	–	–	–	1(1)	2	2	–	–	2	1
15	1-Pentanol[d]	764	764[F]	–	–	1(1)	–	2	2(1)	–	–	–	1
18	3-Methyl-2-butene-1-ol	775	776[B]	–	–	1	1(1)	2(1)	1(1)	–	–	–	–
22	3-Hexen-1-ol	856	857[E]	–	–	–	1(1)	2	1(1)	–	–	–	–
24	1-Hexanol[d]	869	870[D]	–	–	–	1	2	2	–	–	2	1
30	1-Octen-3-ol	981	979[D]	–	–	–	1	2	2	–	–	–	1
33	6-Methyl-5-hepten-2-ol	993	993[E]	–	–	–	–	2	2	–	–	–	1
38	2-Octen-1-ol	1068	1067[G]	–	–	–	1	2	2	–	–	–	1(1)
39	2,6-Dimethyl-7-octen-2-ol	1074	1072[E]	–	–	–	1	2(1)	1(1)	–	–	2(2)	1
42	1-Decanol[d]	1274	1272[E]	–	–	–	1	2	2(2)	–	–	–	1
Aromatic alcohols													
40	2-Phenylethanol[d]	1123	1127[F]	–	–	–	1(1)	2(1)	2(2)	–	–	2(2)	1(1)
Carbonyl Compounds													
3	2-Methylpropanal	556	553[F]	–	–	–	–	2(2)	1(1)	–	–	2(1)	1(1)
7	3-Methylbutanal[d]	653	654[F]	2	3	6	1(1)	2	2	3	–	2	1
9	2-Pentanone[d]	686	686[F]	–	–	4(2)	–	–	–	1	–	–	1
11	3-Pentanone[d]	697	697[D]	2	2	3	1	2	1	–	–	–	1(1)
14	2-Methyl-3-pentanone[d]	749	748[A]	1	–	2(2)	–	–	–	1(1)	–	–	–
19	3-Hexanone[d]	784	784[B]	–	–	3(2)	–	2(2)	2(2)	–	–	–	–
31	6-Methyl-5-hepten-2-one	986	986[D]	1	–	4(4)	1	2	2	–	–	–	–
37	(2*E*)-2-Octenal	1062	1062[F]	–	–	–	1	2(2)	2(2)	–	–	–	–
Alkadienes													
1	Isoprene[d]	508	NEC	2	3	5	1(1)	1	1(1)	4	1	2(1)	1
5	4-Methyl-1,3-pentadiene[d]	636	NEC	2	3	6	1	2	2	3	2(1)	2(1)	1(1)

Table 1 (continued)

Peak n°	Identity	LTPRI exp.	LTPRI lit.[a]	Samples[b, c]									
				H. pulchellus						*H. riojanus*			
				in vivo sampling			Skin sampling			*in vivo* sampling		Skin sampling	
				TA NAT *n*=2	BL NAT *n*=3	BL LAB *n*=6	TA NAT *n*=1	BL NAT *n*=2	BL LAB *n*=2	LR NAT *n*=4	LR LAB *n*=2	LR NAT *n*=2	LR LAB *n*=1
Aromatic hydrocarbons													
8	Benzene[d]	659	661[D]	2	3	6	–	–	–	4	2(1)	–	–
16	Toluene[d]	768	770[D]	2	3	6	1	2	2	4	2	2	1
23	Ethylbenzene[d]	866	866[D]	2	3	6	1	2	2	4	2(1)	2(2)	1(1)
25	p-Xylene[d]	874	874[D]	2	3	6	1	2	2	4	2(1)	2(1)	1
26	Styrene[d]	896	897[F]	–	3	6	1	2	2	4	2(1)	–	1
29	1-Ethyl-methyl-benzene[d]	967	970[F]	2	2	5(2)	1(1)	–	2(2)	4	1(1)	1	–
Esters													
17	Methyl 3-methylbutanoate	776	776[B]	–	–	–	–	–	–	4	1	–	–
21	Methyl 3-methyl-2-butenoate	845	842[H]	–	2	4(2)	1	2	2(1)	3(1)	–	2	1(1)
Nitrogen containing compounds													
32	Benzonitrile[d]	992	988[A]	–	–	4(3)	1	2	2	1(1)	–	2(1)	1(1)
41	2-Isobutyl-3-methoxypyrazine	1171	1167[I]	–	1	–	1	2	–	–	–	1	1(1)
Sulphur containing compounds													
20	4-(Methylsulfanyl)-2-pentene (A)	810	NR	–	–	2(2)	–	–	–	1	1	2(2)	1(1)
27	4-(Methylsulfanyl)-2-pentene (B)	924	NR	2	3	4	1	2(2)	1(1)	1(1)	–	–	–
Monoterpenes													
28	α-Pinene	942	942[D]	–	1	–	–	–	–	1(1)	–	–	–
34	3-Carene	1017	1019[D]	–	–	–	–	–	–	1(1)	–	1(1)	–
35	Limonene[d]	1038	1039[C]	2	–	–	–	–	–	1	–	–	–
36	Eucalyptol[d]	1043	1044[B]	–	1	–	–	–	–	2	–	1	–

[a] *LTPRI lit. sources*: (A) Xu et al. 2003; (B) Larsen and Frisvad 1995; (C) Garcia-Estaban et al. 2004; (D) Vasta et al. 2007; (E) Adams 1995; (F) García et al. (2000); (G) Thakeow et al. 2008; (H) Figuérédo et al. 2006; (I) Zimmermann and Schieberle 2000

Abbreviations: *LTPRI exp* LTPRI experimentally obtained, *LTPRI lit* LTPRI reported in the literature, *NEC* LTPRI reported in the literature from a non equivalent column, *NR* LTPRI not reported in the literature

[b] Results represent the number of frog samples (n) in which the compound was identified considering species, sampling procedure, locality, and specimen condition. *Sample abbreviations*: *TA* Estação Ecológica do Taim (RS, Bz), *BL* Brazo Largo (ER, Ar), *LR* Laguna el Rodeo (Ju, AR), *NAT* wild-caught specimens analyzed within 10 days after capture, *LAB* wild-caught specimens held in lab-controlled conditions for more than 15 months prior to analysis

[c] Numbers in parentheses indicate the number of samples in which the peak had S/N<3. A *dash line* indicates absence of the compound

[d] Structures confirmed by comparison with authentic reference samples

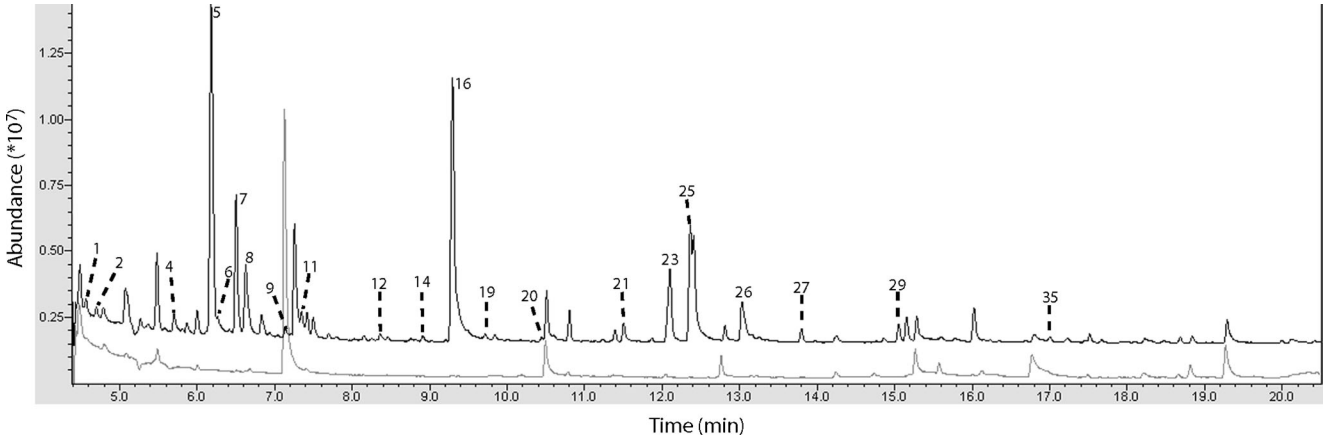

Fig. 2 Total ion current chromatograms of two *in vivo* samplings from the same specimen of *Hypsiboas pulchellus* from Brazo Largo, Argentina, held in laboratory controlled conditions for 15 mo. The grey chromatogram corresponds to the volatile profile of the frog without stimulation, whereas the black trace shows the volatile profile of the stimulated specimen. Identities of peaks are listed in Table 1. Note that peaks with numbers in the black chromatogram are absent in the grey chromatogram

criteria, whereas monoterpenes will be treated separately given their relative importance as a specific class of volatile constituents.

Aliphatic and Aromatic Alcohols

Fifteen aliphatic alcohols (**2**, **4**, **6**, **10**, **12**, **13**, **15**, **18**, **22**, **24**, **30**, **33**, **38**, **39**, **42**) were identified in all samples from both species. Most of these compounds were found in several samples at S/N ≥3. Exceptions were 3-methyl-2-buten-1-ol (**18**) and 3-hexen-1-ol (**22**), which were present at S/N ≥3 in only two samples. The occurrence of these two compounds also varied between the species. They were present in samples of *H. pulchellus*, but absent in samples of *H. riojanus*. Nine compounds, most of them straight-chain primary alcohols, were found only in skin samplings, whereas 2-methyl-3-buten-2-ol, **4** was only present in *in vivo* samplings. The remaining compounds were collected during both sampling procedures. Only one alcohol with an aromatic substituent (2-phenylethanol; **40**) was identified in skin samplings. It occurred in both species at S/N <3. There were no differences in the presence or absence of alcohols between LAB and NAT specimens in *H. pulchellus*. In contrast, five aliphatic alcohols, found in LAB specimens of *H. riojanus*, were absent in NAT specimens.

Carbonyl Compounds

Three aldehydes (**3**, **7**, **37**) and five ketones (**9**, **11**, **14**, **19**, **31**) were identified in all samples. Isovaleraldehyde (3-methylbutanal; **7**) and 3-pentanone (**11**) were found repeatedly in almost all samples, and in most of them at S/N ≥3, whereas 3-hexanone (**19**) and (2*E*)-2-octenal (**37**) were present mostly at S/N <3. 2-Methyl-3-pentanone, **14** was identified only in

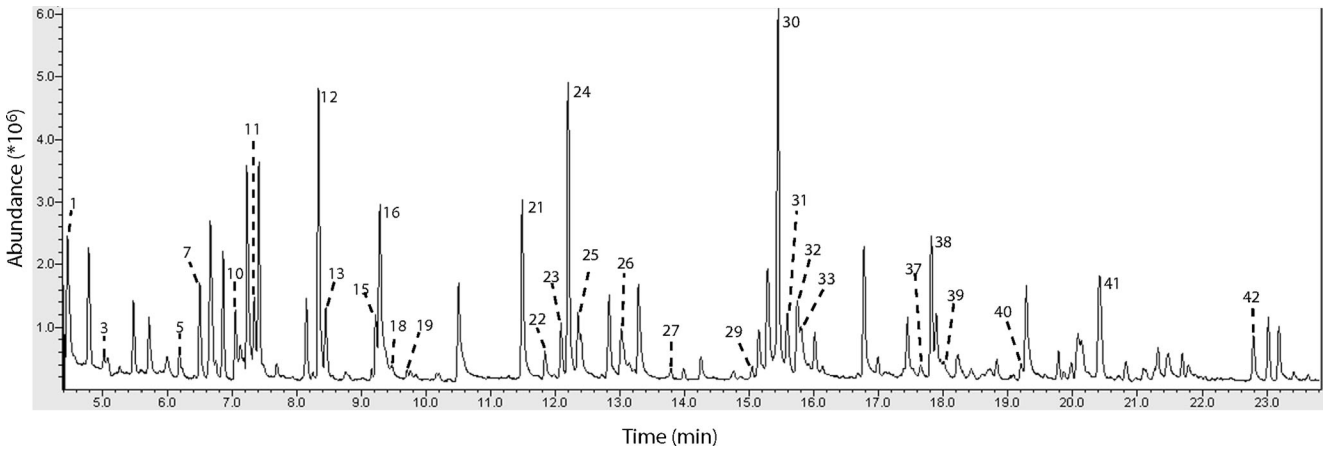

Fig. 3 Total ion chromatogram of skin sampling from the skin of two specimens of *Hypsiboas riojanus* from Laguna el Rodeo, Jujuy, Argentina, which were analyzed within 10 day after being captured. Identities of peaks are listed in Table 1. Most peaks without assignment correspond to impurities that were also present in the blank

in vivo samples, whereas 2-methylpropanal (isobutyraldehyde; **3**) and (2*E*)-2-octenal (**37**), were observed exclusively in skin samples. All volatiles of *H. riojanus* were found also in *H. pulchellus*. In contrast, compounds **19**, **31**, and **37** were present only in *H. pulchellus*. Of these, only 6-methyl-5-hepten-2-one (**31**) was present at S/N ≥3. 2-Pentantone (**9**) and 2-methyl-3-pentanone (**14**) were present in LAB specimens of *H. pulchellus* from BL, but were absent in NAT specimens from the same locality. However, 2-pentantone was present in NAT specimens from TA. In *H. riojanus*, differences in LAB and NAT specimens were registered in two compounds (**11**, **14**), but both were observed only once and at S/N <3.

Alkadienes

Apart from those compounds treated separately as monoterpenes, two alkadienes were identified as isoprene (**1**) and 4-methyl-1,3-pentadiene (**5**). They were found in almost all samples regardless of the sampling procedure, species or specimen conditions. Additionally, in most of the samples they occurred at S/N ≥3.

Aromatic Hydrocarbons

Six aromatic compounds were found (**8**, **16**, **23**, **25**, **26**, **29**) repeatedly in several samples and in most of them at S/N ≥3. Five of these (**16**, **23**, **25**, **26**, **29**) were observed in *in vivo* and skin samplings of both species, whereas benzene (**8**) also occurred in both species, but only in *in vivo* samplings. All aromatic hydrocarbons were found in samples of NAT and LAB specimens, and in both species. In *H. pulchellus,* they occurred in specimens from both localities (BL and TA). Considering that most of these compounds are common solvents in laboratories, it is worth mentioning that none of them was observed in blank analyses, either from the *in vivo* setup or from the 22 ml vial used for skin sampling.

Esters

Two esters (**17**, **21**) were identified in all samples. One of them, methyl 3-methylbutanoate (**17**) with a possible biogenetic background of isoleucine was found in LAB and NAT specimens of *H. riojanus* using *in vivo* sampling, but it was absent in all samples from *H. pulchellus*. The other compound, methyl 3-methyl-2-butenoate (**21**) that differs from the former by the presence of a double bond, possibly representing a *hemi*-terpene, was found in samples from LAB and NAT specimens of both species using *in vivo* and skin sampling. In *H. pulchellus*, it was present in samples from both localities.

Nitrogen Containing Compounds

Two nitrogen containing compounds benzonitrile (**32**) and one pyrazine identified as 2-isobutyl-3-methoxypyrazine (**41**) were observed in this study. Benzonitrile (**32**) was found in *in vivo* and skin samplings of both species. 2-Isobutyl-3-methoxypyrazine (**41**) was found frequently in skin samplings, with the exception of an *in vivo* sample of NAT specimens of *H. pulchellus* from BL. It was present in NAT specimens of *H. pulchellus* from both localities and in NAT and LAB specimens of *H. riojanus*.

Sulfur Derivatives

Two compounds gave very similar mass spectra but different retention times. Their mass spectra showed 92 % similarity with the reference spectrum of 4-(methylsulfanyl)-2-pentene from the NIST library. Considering this evidence, the peaks were assigned to geometric isomers of this compound (A, B; **20**, **27**). These methyl sulfides were found by analyzing *in vivo* and skin samplings from both species, but *in vivo* sampling gave better signal-to-noise ratios. One of the isomers (**20**) was more frequently observed in samples of *H. riojanus*, NAT or LAB specimens using either *in vivo* or skin sampling, whereas it was observed only in two samples from LAB specimens of *H. pulchellus* using *in vivo* sampling. In contrast, the other isomer (**27**) was more frequently observed in NAT and LAB specimens from *H. pulchellus* using both *in vivo* and skin sampling, while it was observed in a single sample from NAT specimens of *H. riojanus.* Their LTPRI are not reported in the literature, and the compounds are not commercially available.

Monoterpenes

Four monoterpenes (**28**, **34**, **35**, **36**) were found in all samples from both species. These compounds were identified as α-pinene (**28**), 3-carene (**34**), limonene (**35**), and eucalyptol (1, 8-cineole, **36**). Of them, α-pinene, limonene, and eucalyptol were observed in both species using *in vivo* sampling, whereas eucalyptol occurred also in a skin sample of *H. riojanus*. 3-Carene was found only in *H. riojanus* using both *in vivo* and skin sampling. In both species, all monoterpenes occurred only in samples from NAT specimens.

SPME-GC/MS Analysis of Dietary and Terraria Samples

Four aromatic hydrocarbons, benzene (**8**), toluene (**16**), ethylbenzene (**23**), and *p*-xylene (**25**), were identified in all the terrarium analyses, whereas isovaleraldehyde (3-methylbutanal; **7**), isoamyl alcohol (3-methyl-1-butanol; **12**), and 2-methylbutanal (present only in dietary samples), were found in the headspace of all dietary samples. Additionally,

toluene (**16**) and 2-methyl-1-butanol (**13**), were observed in two and one dietary sample, respectively. With the exception of 2-methylbutanal, all these compounds also were found in frog samples.

Discussion

Considerations Regarding Extraction Technique

In previous studies of frog volatiles using SPME-GC/MS techniques, Smith et al. (2000, 2003) used an *in vivo* sampling protocol in which the frogs were stressed first by blunt forceps or electrical stimuli, and then were placed in the sample chamber to collect volatiles at room temperature. The *in vivo* sampling method used in our study (Fig. 1) also has been demonstrated to be suitable to sample head space volatiles from living frogs with minimum disturbance. Furthermore, it allows to stimulate the frog in a sealed environment while the fiber is exposed to the headspace, thereby reducing the loss of components. Additionally, as was observed during optimization of the extraction conditions, the internal thermal gradient (*i.e.*, increasing the sample temperature and cooling the fiber) gave better results when collection at room temperature was carried out. This may be partially explained by the air movement along the device due to convection. Although this air movement is not comparable to an air stream in a dynamic HS-SPME (D-HS-SPME), it increased the extraction efficiency of a conventional static HS setup (S-HS). However, the principal explanation may be found in the results presented by Haddadi and Pawliszyn (2009). According to their investigations, recoveries of extraction for all analytes increased when the temperature of the sample increased, due to faster kinetics of desorption from the matrix, whereas, in contrast, the partition coefficient between the fiber and the headspace increased when the fiber was cooled, resulting in increased amounts of trapped volatiles on the fiber.

Differences in the number of observed compounds between sampling procedures (*i.e.*, *in vivo* sampling or skin sampling) may be due to differences in the extraction temperature (35 and 55 °C), and extraction times (20 and 40 min; Merib et al. 2013; Ouyang et al. 2011). There are at least two complementary explanations that may be considered. First, the electrical stimulation applied to living frogs may not be enough to induce the release of some of the volatiles as compared to the action of ultrasound on the isolated skin. Second, some compounds absent in *in vivo* samplings but present in skin samplings may be due to tissue degradation processes. It is worth noting that although both sampling procedures may offer complementary information, the examination of living specimens may be more representative of the true volatile profile (Ouyang et al. 2011). In addition, the non-invasive *in vivo* sampling has some advantages over skin sampling, because frogs can be kept alive and can be repeatedly investigated. These are two important features for the analysis of the effect of different diets and environments in volatile secretions of one single specimen of *H. pulchellus* and *H. riojanus*, as in other species of frogs as well.

Chemical Diversity and Natural Occurrence

This study describes the highest number of volatile compounds identified from the secretion of a single frog species (*i.e.*, 40 in *H. pulchellus* and 37 in *H. riojanus*), and it represents the highest chemical diversity. This higher number of volatiles in *H. pulchellus* and *H. riojanus* in comparison to previous studies may be due to interspecific differences in the volatile profiles, or to differences in extraction techniques; for instance, Poth et al. (2012, 2013) and Starnberger et al. (2013) used conventional solvent extraction, whereas Smith et al. (2000, 2003, 2004a) used S-HS-SPME sampling at room temperature. Current knowledge of the number and diversity of volatile components in different species of anurans is unknown and does not allow one to neglect either option. The extraction procedure described here may be useful for future investigations on frog volatiles and also as a standardized protocol for the comparison of species.

Although most components were common to both species, five compounds, present in samples from *H. pulchellus* were absent in *H. riojanus* [*i.e.*, 3-methyl-2-butene-1-ol (**18**), 3-hexen-1-ol (**22**), (2*E*)-2-octenal (**37**), 3-hexanone (**19**), and 6-methyl-5-hepten-2-one (**31**)]. In contrast, two compounds were found only in samples of *H. riojanus* [*i.e.*, methyl 3-methylbutanoate (**17**) and 3-carene (**34**)]. Excluding those that were observed mostly at S/N <3, two might be considered species-specific compounds: 6-methyl-5-hepten-2-one (**31**) in *H. pulchellus*, and methyl 3-methylbutanoate (**17**) in *H. riojanus*. However, a higher number of samples is needed to validate these inferences.

All alcohols identified in this study are described for the first time in amphibians. They include straight-chain primary alcohols (some of them having a double bond), methyl branched alcohols (primary, secondary, and tertiary), and one aromatic alcohol. There is no previous mention in the literature of the presence of low boiling alcohols (C_5 and C_6) in the secretions of amphibians. However, methyl-branched alcohols with C_8–C_{10} chains have been reported as volatile components of *Mantidactylus femoralis* (Poth et al. 2013).

With the exception of 3-methyl-1-butanol (isoamyl alcohol; **12**), none of the compounds found in samples of *H. pulchellus* and *H. riojanus* was observed in the diet or the terraria. Thus, they likely are associated with the frogs. Although the biosynthesis of all of these compounds is unknown in amphibians, considering the information available for other taxa, they likely

are derived from different biosynthetic pathways. For instance, 1-octen-3-ol (**30**) is associated with an enzymatic cleavage of linoleic acid (Assaf et al. 1995), as well as the two green leaf volatiles, 3-hexen-ol (**22**) and 1-hexanol (**24**; Kiritsakis 1998). 3-Methyl-1-butanol (isoamyl alcohol; **12**), 2-methyl-1-butanol (**13**), and 2-phenylethanol (**40**), would be formed from the degradation of the amino acids leucine, isoleucine, and phenylalanine, respectively (Smit et al. 2005), whereas the hemiterpenes 3-methyl-2-buten-1-ol (prenol; **18**) and 2-methyl-3-buten-2-ol (dimethylvinylcarbinol; **4**) may originate from the mevalonate pathway *i.e.*, from dimethylallyl pyrophosphate (DMAPP; Deneris et al. 1985).

This study is the first report of aliphatic aldehydes and ketones in amphibians. Considering that most of these compounds were absent in the analysis of dietary samples and terraria [exception 3-methylbutanal (isovaleraldehyde; **7**)], their biosynthesis is regarded as associated with the frogs. The presence of isoamyl alcohol and isovaleraldehyde in samples of LAB and NAT specimens of *H. pulchellus* and *H. riojanus,* as well as in the samples of the crickets and mealworms that were used to feed LAB specimens, may imply either that the frogs biosynthesized these compounds, and/or that these compounds accumulate in the frogs from a dietary source. An uptake system that accumulates volatile compounds, specifically monoterpenes (eucalyptol, limonene, and ocimene) and sesquiterpenes (β-caryophyllene) from dietary sources has been demonstrated previously in a hylid species, *L. caerulea* (Smith et al. 2004a).

We describe the presence of two low molecular weight alkadienes, 4-methyl-1,3-pentadiene (**5**) and isoprene (**1**), that are not structurally related, and probably do not originate from the same metabolic pathway. The former is unusual as a natural product, with few references (*e.g.*, Galindo-Cuspinera et al. 2002), whereas isoprene is a common volatile emitted by several plants and bacteria, but has been found also in mice, rats, and humans (King et al. 2012; Sharkey 1996). Like the two hemiterpenes described [(*i.e.*, prenol (**18**) and dimethylvinylcarbinol (**4**)], isoprene is most likely derived from DMAPP (Deneris et al. 1985).

The presence of seven aromatic compounds in several frog samples as well as in samples from terraria, and the absence of all aromatic components in blank analyses, suggests that the terraria represent an environmental source for at least some of these components in *H. pulchellus* and *H. riojanus*. This is supported by the existence of the dermal uptake system of *L. caerulea* (Smith et al. 2004a), and because xenobiotic uptake and bioaccumulation have been reported from different soil and water pollutants in the skin of some amphibian species (Johnson et al. 1999; Linzey et al. 2003; Reynaud et al. 2012).

We describe the presence of two esters that are structurally related differing only by the presence of a double bond. The acid part of methyl 3-methyl-2-butenoate (**21**) may be a hemiterpene formed along the mevalonate pathway (Deneris et al. 1985), whereas the saturated ester may be produced upon hydrogenation of the former, or from leucine.

This study represents the first report of 2-isobutyl-3-methoxypyrazine (**41**) in the volatile secretions of an amphibian species, and to our knowledge, also in vertebrates. In insects, this compound is derived biosynthetically from amino acids and sugar degradation products in some species of Lepidoptera, Coleoptera, Hemiptera, and Orthoptera. Phytophagous members of these orders may sequester pyrazines from their hostplants (Guilford et al. 1987; Moore et al. 1990).

The isomers of 4-(methylsulfanyl)-2-pentene (**20** and **27**) found in the secretions of both species of *Hypsiboas* have not previously been reported as natural products. However, they are structurally related to *E*-2-butene-1-thiol, which is a common component in odorous secretions of skunks (Aldrich 1896; Wood et al. 2003). Another frog species reported to produce sulfur derived odor, more specifically mercaptan-like odor, is *Aromobates nocturnus* (Myers et al. 1991). The fact that organosulfur compounds may be perceived at ppb concentrations in air (Aldrich 1896) may help explain why individuals, such as small frogs emit odoriferous secretions that are perceived from more than four meters away (Brunetti pers. obs.).

Four monoterpenes were identified only in samples of NAT specimens, suggesting that these compounds would accumulate in the skin of *H. pulchellus* and *H. riojanus* from environmental sources. This evidence as well as the findings that were discussed in previous paragraphs, suggest that these species developed an efficient system to sequester different volatile components from a variety of environmental sources.

Biological Function

The volatile secretions in amphibians have been related to several functions (*e.g.*, sexual pheromones, chemical defense) but for most of them experimental evidence is lacking. Given that the characteristic smell of *H. pulchellus* and *H. riojanus* is released under stress situations (Brunetti pers. obs.; Gallardo 1958), it is most likely to act for defense against predators. Such a strategy would include it as a primary repellent against predators (Smith et al. 2003) and/or olfactory aposematism (Sazima 1974). Odoriferous secretions of some anurans have been described as noxious and unpleasant (Myers et al. 1991; Smith et al. 2004b), but they have not yet been demonstrated to be toxic. Smith et al. (2003) have doubted that under natural conditions the intensity of the odor in *L. caerulea* may reach levels high enough to act as primary repellents against predators. This observation led Smith et al. (2003) to propose that odoriferous secretions might act as an aposematic odor, signaling toxicity or unpalatability through the formation of a conditioned reflex (Sazima 1974; Smith et al. 2003). Some

factors that would contribute to the formation of a conditioned aversion to the odor of *H. pulchellus* and *H. riojanus* are the emission of volatile secretion in a predation context (as it occurs only upon handling), and the unique nature of some of these volatile components, particularly the isomers of 4-(methylsulfanyl)-2-pentene, because of their apparent source-specificity.

Adult anurans that are attacked by predators usually emit distress calls, and it has been proposed that they might serve to warn neighbors (Wells 2007). During our preliminary predation tests, we observed that individuals of *H. pulchellus* that are seized by a snake emitted a distress call along with odoriferous secretions. Considering increasing evidence on the use of multiple signals in amphibians (*e.g.*, Grafe et al. 2012; Starnberger et al. 2014), and that multimodal signaling also may act in a the context of predation (Hölldobler 1999), the simultaneous release of chemical and acoustic signals in *H. pulchellus* (and likely *H. riojanus*) needs to be examined in behavioral experiments within a multiple signal context.

Alternatively, and considering other proposed functions for volatiles of anurans, but without excluding the former possibilities, volatile secretions from *H. pulchellus* and *H. riojanus* might act as chemical camouflage (*e.g.*, Smith et al. 2004a), or mosquito repellents (Williams et al. 2006). New investigations from different fields (*e.g.*, chemistry, behavior, physiology) on the volatiles of amphibian secretions will certainly help to answer some of the questions associated with their biological functions – of which little is known today.

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