



# Take time to smell the frogs: vocal sac glands of reed frogs (Anura: Hyperoliidae) contain species-specific chemical cocktails

IRIS STARNBERGER<sup>1\*</sup>, DENNIS POTH<sup>2</sup>, PARDHA SARADHI PERAM<sup>2</sup>, STEFAN SCHULZ<sup>2</sup>, MIGUEL VENCES<sup>3</sup>, JETTE KNUDSEN<sup>4</sup>, MICHAEL F. BAREJ<sup>5</sup>, MARK-OLIVER RÖDEL<sup>5</sup>, MANFRED WALZL<sup>1</sup> and WALTER HÖDL<sup>1</sup>

<sup>1</sup>Department of Integrative Zoology, University of Vienna, Althanstrasse 14, Wien 1090, Austria

<sup>2</sup>Institute for Organic Chemistry, Technical University of Braunschweig, Braunschweig, Germany

<sup>3</sup>Zoological Institute, Technical University of Braunschweig, Braunschweig, Germany

<sup>4</sup>Department of Biology, Lund University, Lund, Sweden

<sup>5</sup>Museum für Naturkunde Berlin, Leibniz Institute for Research on Evolution and Biodiversity, Berlin, Germany

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Males of all reed frog species (Anura: Hyperoliidae) have a prominent, often colourful, gular patch on their vocal sac, which is particularly conspicuous once the vocal sac is inflated. Although the presence, shape, and form of the gular patch are well-known diagnostic characters for these frogs, its function remains unknown. By integrating biochemical and histological methods, we found strong evidence that the gular patch is a gland producing volatile compounds, which might be emitted while calling. Volatile compounds were confirmed by gas chromatography–mass spectrometry in the gular glands in 11 species of the hyperoliid genera *Afrivalus*, *Heterivalus*, *Hyperolius*, and *Phlyctimantis*. Comparing the gular gland contents of 17 specimens of four sympatric *Hyperolius* species yielded a large variety of 65 compounds in species-specific combinations. We suggest that reed frogs might use a complex combination of at least acoustic and chemical signals in species recognition and mate choice. © 2013 The Authors. Biological Journal of the Linnean Society published by John Wiley & Sons Ltd on behalf of The Linnean Society of London, *Biological Journal of the Linnean Society*, 2013, 110, 828–838.

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## INTRODUCTION

In the animal kingdom male signals usually offer information about the individual's condition, underpinning beneficial traits that might be passed on to the next generation. Females recognize these signals and use them in mate choice (Zahavi, 1975). In most anuran amphibians advertisement calls are the predominant signal in inter- and intrasexual communi-

cation (Ryan, 1985; Gerhardt & Huber, 2002; Dorcas *et al.*, 2010). The male advertisement call attracts conspecific females and signals a readiness to defend territories against conspecific males (Duellman & Trueb, 1986). Hence calling behaviour plays a vital role in reproductive success, and is essential for sexual selection. The calling behaviour of frogs and toads has been the subject of a large number of studies, whereas other channels of communication possibly used in a reproductive context (e.g. seismic, visual, or chemical communication) in these organisms have been almost completely neglected (Taylor,

\*Corresponding author. E-mail: iris.starnberger@univie.ac.at

Buchanan & Doherty, 2007; Coleman, 2009), but see the review by Hödl & Amézquita (2001) and two studies on seismic communication in frogs (Lewis *et al.*, 2001; Caldwell *et al.*, 2010).

THE ANURAN VOCAL SAC AND ITS ROLE  
IN COMMUNICATION

In males of most anuran species the vocal sac is associated with producing advertisement calls. The main function of the vocal sac is to recycle the air coming from the lungs during calling. Furthermore, it also minimizes the loss of sound energy by decreasing the impedance mismatch between the frog's body cavity and its environment, increases the call rate, and distributes sound waves omnidirectionally (Bucher, Ryan & Bartholomew, 1982; Rand & Dudley, 1993; Pauly *et al.*, 2006). Because the vocal sac inevitably moves while a male is calling, it might send a fixed composite signal (*sensu* Partan & Marler, 2005) consisting of the acoustic signal component and a visual trait, with increased detectability arising from the movement (Endler & Thery, 1996; Fleishman *et al.*, 1998; Rosenthal, Rand & Ryan, 2004; Taylor *et al.*, 2008). However, the way in which multimodal signals interact is species specific, and might differ immensely. Hirschmann & Hödl (2006) showed that in *Phrynobatrachus krefftii* Boulenger, 1909 the conspicuous yellow vocal sac functions as a signal in male–male agonistic interactions, even without calls within the human audible range being emitted. As chemosignals can usually be produced at low costs (Hedin, Maxwell & Jenkins, 1974), the use of pheromones might be a widespread phenomenon in anuran species recognition and mate choice also, but to date the possibility of chemical communication in anurans has not been considered by most studies (Waldman & Bishop, 2004; Belanger & Corkum, 2009), probably because of the more conspicuous signal modalities present to the human observer (e.g. acoustic and visual), or because of the overly costly and elaborate analyses necessary to investigate pheromones.

CHEMICAL COMMUNICATION IN AMPHIBIANS

A large number of amphibians use chemical cues for navigation (Sinsch, 1990; Schulte *et al.*, 2011) and predator detection (Flowers & Graves, 1997), both in aquatic and terrestrial environments. This leads to the assumption that many species may also have the physiological and anatomical ability to produce and detect hetero- and conspecific chemical signals (Byrne & Keogh, 2007; Woodley, 2010; Hamer, Lemckert & Banks, 2011). In aquatic and terrestrial urodeles there are many well-known cases of chemical communication in a sexual context. For instance, male newts of

the genus *Lissotriton* release a bouquet, use their tail to fan it towards a female, and thus elicit responses necessary to coordinate spermatophore transfer (e.g. Malacarne & Giacoma, 1986; and see Treer *et al.*, 2013). There are also a few reports of aquatic sex pheromones (i.e. molecules used for communication between conspecific animals in an aquatic environment) in anurans. In African clawed-frogs (*Hymenochirus* sp.), females tested in Y-maze experiments showed a clear preference for water containing homogenized male post-axillary breeding glands, or for water previously containing live males (Pearl *et al.*, 2000). Wabnitz *et al.* (1999) found that female *Litoria splendida* Tyler, Davies & Martin, 1977 are attracted towards the male by splendipherin, an aquatic pheromone produced only by males in glands found on the head. The chemicals identified in *L. splendida*, *Hymenochirus* spp., as well as in newts and salamanders, are peptides, and can therefore only be spread in water or through direct contact (Rajchard, 2005; Houck, 2009). As a result of their direct contact with the female during amplexus, there are a considerable number of publications speculating on the possible use of skin glands, present in terrestrial male anurans, in chemical communication in a sexual context (e.g. Thomas, Tsang & Licht, 1993; Rödel *et al.*, 2003; Lenzi-Mattos *et al.*, 2005). But to the best of our knowledge, there are only four reported cases of volatile pheromone communication in terrestrial amphibians. Male American toads [*Anaxyrus americanus* (Holbrook, 1836)] show orientation towards female chemical cues in a Y-maze set-up (Forester & Thompson, 1998). Korbeck & McRobert (2005) report, that poison frogs [*Dendrobates auratus* (Girard, 1855)] are attracted towards conspecifics of the opposite sex by olfactory cues. In the Australian toadlet *Pseudophryne bibronii* Günther, 1859, males call hidden in the leaf litter at night and secrete an odorous mucus produced by dorsal, axillary, and postfemoral skin glands, which is likely to help females in close-range mate localization (Byrne & Keogh, 2007). In a recent study on mantellid frogs, Poth *et al.* (2012) provided the first direct evidence for the occurrence of volatile compounds acting as pheromones in anurans. These compounds are emitted in species-specific cocktails from the prominent femoral glands that characterize the males of these endemic Malagasy frogs (Vences *et al.*, 2007; Poth *et al.*, 2012; Poth *et al.*, 2013).

AN EXTRAORDINARY VOCAL SAC STRUCTURE  
IN HYPEROLIIDS

The anuran family Hyperoliidae (reed frogs) is common in sub-Saharan Africa, Madagascar, and the Seychelles, and comprises over 200 species in 18 genera, of which *Hyperolius* is the most species rich



**Figure 1.** From top left to bottom right: sympatric male individuals of *Hyperolius cinnamomeoventris*, *Hyperolius kivuensis*, *Hyperolius viridiflavus*, and *Hyperolius lateralis* with inflated vocal sacs. The prominent gular patch is visible in all pictures. (Photos by I. Starnberger and W. Hödl, taken at Kibale Forest National Park, Uganda).

(Frost, 2012). Within this clade there is substantial variation in coloration, morphology, and reproductive modes, but males of all reed frog species share a common feature: a prominent gular patch on the vocal sac (Fig. 1). Schiøtz (1967) described the gular patches as ‘protective flaps’, suggesting a function as a robust shield for the thin skin of the vocal sac. Drewes (1984) conducted a broad survey, during which he found different variations concerning the position and the shape of the gular patches in reed frogs, and described them as glands of which the ‘actual function remains obscure’. Some genera have gular patches but lack a vocal sac, and Perret (1961) suggested chemical communication in *Acanthixalus spinosus* (Buchholz & Peters, 1875). Rödel *et al.* (2003) provided further evidence for possible chemical communication in two apparently mute hyperoliid species from West and Central Africa [*Acanthixalus sonjae* Rödel, Kosuch, Veith & Ernst, 2003 and *Acanthixalus spinosus* (Buchholz & Peters, 1875), but see Amiet, 1972], in which males have two gland-like structures on the throat.

The present study aims to shed light onto the structure and function of the conspicuous hyperoliid gular patch and its potential to bear a function in species recognition, mate choice, and also as a consequence in speciation. We first performed histological examinations to reveal the internal structure of the gular patch. Furthermore, we searched for volatile substances in the gular patch tissue of 11 members of

the family Hyperoliidae that would enable airborne chemical communication. Finally, we tested the gular patches of four syntopic species of *Hyperolius* for the possible presence of species-specific cocktails of volatile substances, which would offer these frogs the opportunity of chemical species recognition. This study aims to lay the foundation for future experimental studies to empirically test the function of the gular patch.

## MATERIAL AND METHODS

### COLLECTION AND MOLECULAR IDENTIFICATION OF SPECIMENS

To test the possibility of species recognition via chemical cues, we collected syntopic specimens in Kibale Forest National Park, Uganda, near the Makerere University Biological Field Station (0°33′41.4786″N, E 30°21′23.6838″). Free-living adult specimens of *Hyperolius cinnamomeoventris* Bocage, 1866 ( $n = 3$ ), *Hyperolius kivuensis* Ahl, 1931 ( $n = 3$ ), *Hyperolius lateralis* Laurent, 1940 ( $n = 3$ ), *Hyperolius viridiflavus* Dumeril & Bibron, 1841 ( $n = 8$ ), and *Phlyctimantis verrucosus* (Boulenger, 1912) ( $n = 1$ ) were caught in the field at night (when the males were calling). The frogs were sedated by the application of a small quantity of benzocaine on the back, which is absorbed through the frog’s skin, and then killed by an overdose of the same substance in the field laboratory. Tissue from the vocal sac was removed and immediately fixed, either in

formalin for histology (see below) or in methanol for chemical analysis. The latter samples were stored in 1 mL gas chromatography vials sealed with Teflon-coated caps to prevent modification or evaporation of chemical compounds. In addition, small quantities of muscle tissue for DNA analysis were preserved in absolute ethanol. To allow for a broader biochemical survey, specimens of additional hyperoliid species were collected in Cameroon (genera *Afrixalus* and *Hyperolius*) and Madagascar (*Heterixalus*) in the framework of other research projects, and fixed using the same protocols as in Kibale Forest. The species collected in Cameroon were identified based on morphological traits (Amiet, 2012). The *Heterixalus* specimens collected in Madagascar could be clearly distinguished by their species-specific calls, as only calling males were sampled. In three specimens of *Heterixalus* spp. we also examined samples from the skin of the belly and the skin of the vocal sac after excision of the gular gland. A list of voucher specimens is provided in Table S3.

Because many East African *Hyperolius* species are morphologically similar to each other, the identification of all specimens collected in Kibale Forest was confirmed by DNA sequences. DNA extraction from ethanol-preserved tissues, polymerase chain reaction (PCR), and direct sequencing of a DNA fragment of the mitochondrial 16S rRNA gene (~500 nucleotides) followed standard methods (e.g. Vences *et al.*, 2003). We used the primers 16S-AL and 16S-BH for PCR, and primer 16S-AL for sequencing (see Vences *et al.*, 2003 for primer sequences and thermocycling protocols). All newly determined sequences were deposited in GenBank (accession numbers GenBank KF447778–KF447836). Haplotypes determined from specimens of each of the four species (*H. cinnamomeoventris*, *H. kivuensis*, *H. lateralis*, and *H. viridiflavus*) were almost invariable within species and highly divergent between species, thus confirming that the specimens indeed belong to four distinct species. BLAST searches against sequences in GenBank confirmed the species identification of each of the four clusters.

#### COMPOUND EXTRACTION AND ANALYSIS

The gular patch tissue samples stored in methanol were concentrated and directly analysed by gas chromatography–mass spectrometry (GC-MS) methods. GC-MS analysis was used to investigate the gland constituents, as they are presumably released during calling and should therefore be volatile. The analyses were performed on an Agilent 7890A GC system fitted with an HP-5MS-fused silica capillary column (30 m, 0.25 mm i.d., 0.25 µm film; J&W Scientific), connected to an Agilent 5975C inert mass detec-

tor using the following method: 5 min at 50 °C, then increasing with 10 °C min<sup>-1</sup> to 320 °C, operated in splitless mode (60-s valve time). The detected compounds were characterized by their molecular mass, their base peak, and their retention index (RI). Most of the compounds were assigned to one of the four substance classes most abundant in the samples (sesquiterpenes, fatty acid esters, macrolides, alcohols), based on their characteristic fragmentation patterns. The structures of some volatile compounds were identified by the comparison of their mass spectra and retention indices with data from the literature.

#### HISTOLOGY

For histological analyses specimens of *H. viridiflavus* ( $n = 2$ , both green morphs) were killed as described above and fixed in 10% neutral buffered formalin, as described by Carson (1997), in the field lab. The samples were submerged in Bouin's fixative and after 24 h transferred to ethanol (70%). The samples were dehydrated in a graded series of ethanol (70, 80, and 90%) and embedded in paraffin. The tissue was cut at 5 µm using a rotary microtome (Leica RM 2265). The 5 µm series was stained using Heidenhain's AZAN stain for general histology. We used only one species for detailed histological analysis, because a pilot study showed that the gland tissue anatomy does not vary considerably between closely related *Hyperolius* species (I. Starnberger, unpubl. data).

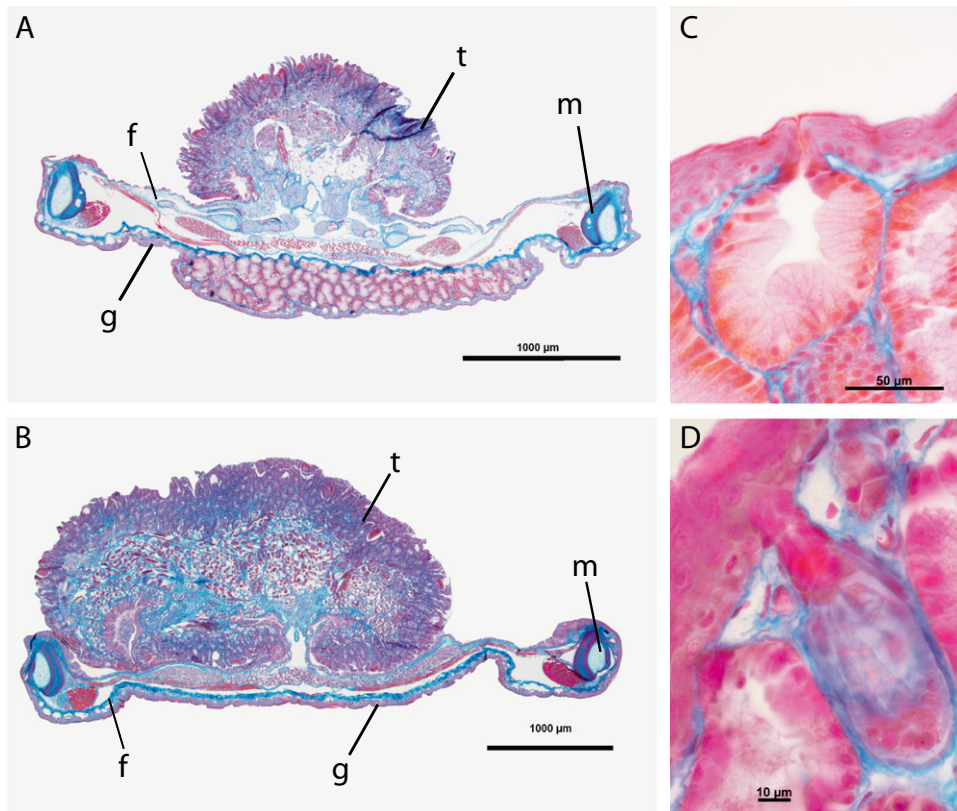
#### STATISTICAL ANALYSES

To graphically visualize variation in individual compound profiles within and among species of *H. cinnamomeoventris*, *H. kivuensis*, *H. lateralis*, and *H. viridiflavus*, based on the untransformed presence/absence of compounds (Table S1), we used non-metric multidimensional scaling (nMDS), based on Bray–Curtis dissimilarity matrices. To quantify the difference in compound composition between sympatric species we used a one-way analysis of similarity (ANOSIM) with Bray–Curtis distance measure (as in Russo *et al.*, 2008). Statistical analyses were performed with SPSS 20.0 and PAST 2.17 (see Hammer, Harper & Ryan, 2001).

#### RESULTS

##### STRUCTURE OF GULAR PATCH AND GULAR GLAND

Histological analyses revealed that the conspicuous gular patch found on the vocal sac of male reed frogs is a gland complex consisting of different gland types. The hyperoliid gular patch is a hypertrophied area extending far into the vocal sac cavity, and is approximately ten times thicker than the surrounding vocal



**Figure 2.** Cross sections (6- $\mu$ m thick; AZAN stain) of the lower jaw of a male (A) and a female (B) *Hyperolius viridiflavus*, showing the tongue (t), the mandibular arch (m), the floor of the mouth (f), and the gular skin (g). The male's gular skin is extremely hypertrophied and forms a tubular gland complex, which is missing in the female. Details of tubular glands reveal collective pools near the surface of the gland complex with narrow ducts leading to the outside (C), and myocytes, probably used to discharge the gland (D). The tongue size difference between the male and the female is linked to the female's larger body size and the fact that the jaws of the two specimens were cut in slightly different areas.

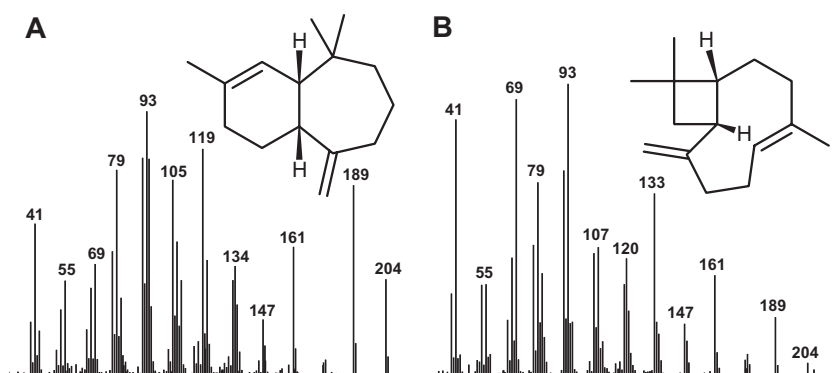
sac tissue (Fig. 2A, C and D). The gular patch is situated in the stratum spongiosum within the dermis, and comprises three different gland types. We hereafter use the term 'gular gland' to refer to this macrogland structure. A vast part of the tissue is made up of glands consisting of densely packed, highly prismatic cells with a central nucleus, which form tubular ducts leading to a basin directly underneath the skin surface (Fig. 2A, C and D). Secretions collected in the basin can be released onto the epidermis via a narrow duct. The collective basins are surrounded by myocytes, which most likely compress the gland to push the secretion through the narrow duct (Fig. 2A, C and D). The gular gland is made up by separate units of tubular glands, subdivided by thin layers of connective tissue, each with a separate secretion channel. Furthermore, there is a strand of connective tissue conjoining the gular patch with the diaphragma oris. The vocal sac tissue surrounding the gular patch contains both granular and mucous glands, but no tubular gland tissue.

#### CHEMICAL COMPOUND SCREENING IN HYPEROLIID FROGS

We performed a biochemical screening of members of four hyperoliid genera, all characterized by a prominent gular patch (Table 1). The chemical survey revealed that sesquiterpenes are present in the gular patches of all genera sampled (*Afrixalus* spp., *Heterixalus* spp., *Hyperolius* spp., and *Phlyctimantis* sp.), with up to 13 different compounds per individual, suggesting that in all of these taxa the patches are made up by a gular gland structure. Fatty-acid esters were present in all *Hyperolius* samples, with 12–14 compounds per individual, and in *Heterixalus alboguttatus* (Boulenger, 1882) (seven compounds) and in *Afrixalus lacteus* Perret, 1976 (one compound). Furthermore, we found a small number of macrolides in two *Hyperolius* species and in *Afrixalus paradorsalis* Perret, 1960. In the samples of *H. cinnamomeoventris* and *H. viridiflavus* a number of unidentified alcohols were present. In

**Table 1.** Total number of compounds of each substance class present in the samples of the hyperoliid species examined

Genus	Species	No. of samples	Sesquiterpenes	Fatty acid esters	Macrolides	Alcohols	Total no. of compounds
<i>Hyperolius</i>	<i>kivuensis</i>	3	13	12			28
<i>Hyperolius</i>	<i>lateralis</i>	3	3	12			18
<i>Hyperolius</i>	<i>cinnamomeoventris</i>	3	7	12	2	1	25
<i>Hyperolius</i>	<i>viridiflavus bayoni</i> (brown morph)	5	2	14	1	2	24
<i>Hyperolius</i>	<i>viridiflavus bayoni</i> (green morph)	3	4	14	1	1	23
<i>Hyperolius</i>	<i>ademetzi</i>	1	11				12
<i>Afrixalus</i>	<i>lacteus</i>	3	1	1			5
<i>Afrixalus</i>	<i>laevis</i>	3	2				5
<i>Afrixalus</i>	<i>paradorsalis</i>	3	10		1		14
<i>Phlyctimantis</i>	<i>verrucosus</i>	1	10				11
<i>Heterixalus</i>	<i>alboguttatus</i>	6	13	7			26
<i>Heterixalus</i>	<i>betsileo</i>	3	6				9

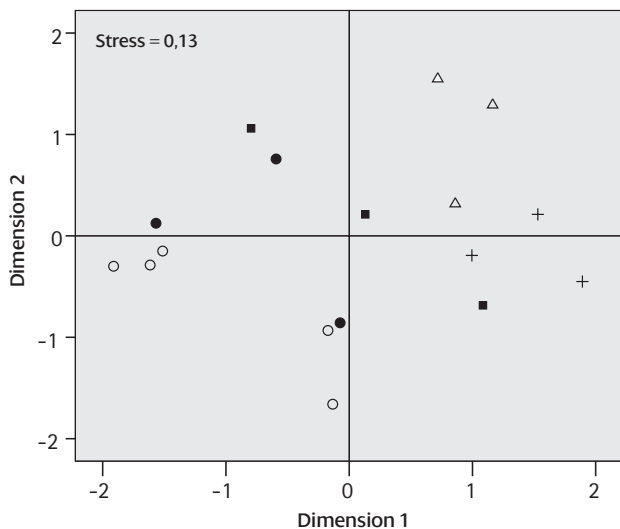

**Figure 3.** Mass spectra of the terpenes identified from *Hyperolius cinnamomeoventris* ( $\alpha$ -himachalene, A) and from *Hyperolius kivuensis* [2-epi-(*E*)- $\beta$ -caryophyllene, B]. The structures were confirmed by comparison of the mass spectrometric data and the retention index with those from the literature (Adams, 1995).

*Heterixalus* spp. none of the three control samples of skin from the belly and skin from the vocal sac yielded any volatile compounds, confirming that these are produced and secreted by the gular gland only.

Although the structures of most of the volatiles detected in the gular patch extracts are still unknown, some compounds could be identified. Figure 3 shows the mass spectra and structures of two sesquiterpenes detected in the gular patch extracts of *H. kivuensis* and *H. cinnamomeoventris*. The mass spectrometric data and the retention index of  $\alpha$ -himachalene and 2-epi-(*E*)- $\beta$ -caryophyllene matched those reported by Adams (1995). The identification of the structures of other sesquiterpenes and of the detected alcohols and macrolides will be pursued in the future.

#### CHEMICAL COMPOUND DIFFERENTIATION IN FOUR SYNTOPIC *HYPEROLIUS* SPECIES

In total, 65 compounds were recorded from gular patch tissue of the 17 males examined from four *Hyperolius* species collected at Kibale. Most compounds could be assigned to a structural class, of which sesquiterpenes and fatty acid esters were the most abundant. Overall, the chemical profiles were different between species (ANOSIM:  $P = 0.0028$ ,  $R = 0.5$ ), which is illustrated by the fact that only a small portion of the terpenes was shared by the different syntopic species, and most of them were characteristic for one species (Table S2). Multidimensional scaling based on the presence/absence of compounds in the individual samples resulted in clustering (Fig. 4), confirming that each species



**Figure 4.** Non-metric multidimensional scaling (nMDS) plot of *Hyperolius* chemical compound profiles among four sympatric species. The nMDS plot is based on the presence/absence of 65 compounds in the gular patch tissue samples (Table S1). Each symbol represents an individual frog. Symbol shape refers to species identity ( $\Delta$ , *Hyperolius kivuensis*; +, *Hyperolius cinnamomeoventris*;  $\circ$ , *Hyperolius viridiflavus*, brown morph;  $\bullet$ , *H. viridiflavus*, green morph;  $\blacksquare$ , *Hyperolius lateralis*).

has a characteristic cocktail of compounds in its gular glands, despite individual variation (nMDS: stress = 0.1). All four species were largely separated, but the chemical bouquets of *H. cinnamomeoventris* and *H. viridiflavus* differed the most ( $P = 0.012$ ). *Hyperolius cinnamomeoventris* and *H. lateralis* had chemical cocktails that clustered in between the two other species, but still did not overlap. The highest variation of chemical composition between individuals was found in *H. viridiflavus*, with one individual even clustering with *H. lateralis*. The other specimens of *H. viridiflavus* formed two clusters that seem to correspond to body coloration (i.e. different colour morphs).

## DISCUSSION

### STRUCTURE AND FUNCTION OF THE GULAR PATCH

The present study shows that the colourful gular patch found on the vocal sac of reed frogs (Hyperoliidae) is a gland complex (here named the gular gland), with a histological structure that is, in general, similar to other macroglands of anuran amphibians (Thomas *et al.*, 1993), including the pheromone-secreting femoral glands of mantellid frogs (Vences *et al.*, 2007). In urodeles, a similar structure has been described and analysed in detail from the cloacal gland of the salamander *Eurycea*

*lucifuga* Rafinesque, 1822 (Sever, 1989; Hamlett, Strecker & Trauth, 1998). *Eurycea* males develop barrel-shaped caudal courtship glands surrounded by round mucous and granular glands, which considerably increase in size during the breeding season. The courtship glands of these salamanders consist of columnar cells with basal nuclei, which form two short tubular ducts leading directly to a duct opening ending on the surface of the epidermis. In the case of *E. lucifuga*, male pheromones are transmitted through the skin of the female during direct contact, as in other plethodontids. Because of the general structural similarity with other amphibian glands involved in pheromone production, the gular gland of hyperoliids is likely to serve a similar function; however, the mode of transmission remains unclear so far, and will be experimentally tested in future studies.

One possibility is direct transmission of chemical compounds from male to female during amplexus, when the male's gular region is tightly pressed onto the female's back, as in salamanders. For instance, in newts of the North American genus *Notophthalmus*, the male rubs his chin on the female's head to transmit courtship pheromones (Hilton, 1902). In this scenario, female reed frogs could chemically ascertain the conspecificity and potentially the attractiveness of the amplexing male, and potentially delay oviposition if amplexed by a male deemed unsuitable.

A second possible mode of pheromone transmission would be the production of volatile substances that are emitted while a male is calling and circulate in the air to attract females, and/or to keep rival males at a distance. In *Acanthixalus spinosus* (Schmitz, Euskirchen & Böhme, 1999) and *Acanthixalus sonjae* (M.-O. Rödel, pers. observ.) there is a distinctive smell from the gular glands that is discernible to the human observer, and the presence of volatile sesquiterpenes among the compounds identified in reed frog gular glands makes this alternative conceivable, calling for future behavioural studies testing these hypotheses.

### CHEMICAL COMPOUND DIVERSITY IN REED FROGS

Our study confirmed the presence of volatile compounds in the gular patches of representatives of a subset of the 19 hyperoliid genera known at present. The data set includes members of four genera, including the most species-rich ones (*Hyperolius*, *Afixalus*, and *Heterixalus*). As tissue samples of museum specimens conserved in ethanol or formalin cannot be used for the chemical screening, fresh additional samples are needed to ascertain the presence of such compounds in the remaining genera.

The results from four syntopic *Hyperolius* species at Kibale Forest suggest that substances produced in

the gular patch might play a role in species recognition. In our sampling of these four *Hyperolius*, sesquiterpenes were not usually shared among different species and thus could play a role in the chemical communication of the Hyperoliidae. Fatty acid esters were present throughout samples of all of the four species, and are therefore not species specific. This broad occurrence, their low volatility, and the fact that fatty acid esters are commonly found in anuran tissues makes their potential role in airborne chemical communication unlikely. The species-specific occurrence of the alcohols and the macrolides, only found in the samples of *H. viridiflavus* and *H. cinnamomeoventris*, are a good indication that those volatiles together with the terpenes may be used to create species-specific cocktails of chemicals. Because alcohols and macrolides are known to be used as volatile pheromones by Malagasy mantellid frogs (Poth *et al.*, 2012), they may also play a role in the chemical communication and speciation of the Hyperoliidae. In *H. viridiflavus* the two colour morphs (brown and green) appear to form largely separated groups (Fig. 4). This result might point towards a possible incipient divergence within the so-far unresolved ‘*viridiflavus*’ species complex, although in our analysis, specimens of both morphs had identical haplotypes in the mitochondrial *16S* gene.

REED FROGS MIGHT PRODUCE TRIMODAL SIGNALS WHILE CALLING

Reed frogs can often be found calling in mixed choruses together with closely related species (Schjötz, 1999; e.g. Lötters *et al.*, 2004). We hypothesize that for female reed frogs, advertisement calls alone might not be sufficient to precisely locate and identify a conspecific male in a mixed-species chorus, and in dense vegetation such as reeds. Hödl (1977) and Martins, Almeida & Jim (2006) showed that in the Neotropics, males in heterospecific choruses use calling site segregation (i.e. different plant species and different calling heights) to facilitate female approach to conspecific males. The four syntopic reed frog species studied herein called simultaneously in the same swamp, without any obvious spatial or temporal segregation, although spatial segregation is not uncommon in hyperoliids (Rödel, Lampert & Linsenmair, 2006; Sinsch *et al.*, 2012). Wilbur, Rubenstein & Fairchild (1978) speculated that the ‘vocal sac pouch’ in the hyperoliid *Kassina senegalensis* (Dumeril & Bibron, 1841) plays a role in attracting females and/or in setting up breeding territories. From our data, we suggest that along with macrolides and alcohols, sesquiterpenes in particular might be the prime components in the chemical sig-

nalling of hyperoliids. Sesquiterpenes were found in the gular glands of all species examined, and as they are volatile they could therefore act as airborne pheromones released while calling.

As acoustic signals such as individual anuran advertisement calls are difficult to localize in a chorus situation (Bee & Micheyl, 2008; Richardson *et al.*, 2010), or in environments (e.g. dense vegetation) that hamper the propagation and transmission of acoustic signals (Wells & Schwartz, 1982; Kime, Turner & Ryan, 2000; Boeckle, Preininger & Hödl, 2009), the differential colour of vocal sacs and gular patches of reed frogs might help in species recognition. A surprisingly high contrast between the gular patch and the surrounding vocal sac skin makes the gland stand out from its background, and might serve as a visual cue facilitating the localization of a male calling in dense vegetation (I. Starnberger, pers. observ.). In addition, ‘chemical cocktails’ might be used for close-range species identification. Such chemical signals might also contain cues on a male’s ‘good genes’ and genetic compatibility, and might therefore influence a reproductive female in mate choice (reviewed in Johansson & Jones, 2007).

CONCLUSION

We propose that reed frogs use a combination of acoustic and chemical signals to enhance their ability to detect conspecifics within the dense multi-species breeding aggregations typical for hyperoliid frogs. Furthermore, it is likely that the vocal sac plays a role in visual signalling by its conspicuousness. Future experimental studies thus might characterize hyperoliids as a highly attractive model for multimodal communication. Nevertheless, there are several hyperoliid species where males lack an inflatable vocal sac but still have one or two gular glands, and several of these species seem to be mute and might therefore fully rely on chemical communication. Chemical cocktails found in the gular gland are species specific, and might even help to resolve taxonomic issues in this species complex.

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### SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Table S1.** (a) First part of list of absence and presence data for chemical compounds in tissue samples of 17 specimens belonging to four reed frog species (*Hyperolius*). (b) Second part of list of absence and presence data for chemical compounds in tissue samples of 17 specimens belonging to four reed frog species (*Hyperolius*).

**Table S2.** Occurrence of volatile compounds in the gular patch extracts from 17 individuals belonging to four *Hyperolius* species.

**Table S3.** Voucher numbers of specimens used in the biochemical survey.