



universität
wien

DISSERTATION / DOCTORAL THESIS

Titel der Dissertation / Title of the Doctoral Thesis

„The role of the vocal sac in multimodal signalling
- an explorative study in African reed frogs“

verfasst von / submitted by

Mag.rer.nat. Iris Starnberger

angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Doctor of Philosophy (PhD)

Wien, 2018 / Vienna, 2018

Studienkennzahl lt. Studienblatt /
degree programme code as it appears on the student
record sheet:

A 094 437

Dissertationsgebiet lt. Studienblatt /
field of study as it appears on the student record sheet:

Biologie

Betreut von / Supervisor:

ao. Univ-Prof. i.R. Mag. Dr. Walter Hödl

Mitbetreut von / Co-Supervisor:

Dr. Doris Preininger

Acknowledgements

"In jede hohe Freude mischt sich eine Empfindung der Dankbarkeit."

(Marie Freifrau von Ebner-Eschenbach)

First of all I would like to thank my "Doktorvater" Walter Hödl for sharing his research idea so generously, for kindling the spark of my fascination for (tropical) frogs and for the countless unforgettable experiences I gained while pursuing this fascination under his guidance.

I am extremely grateful to Doris Preininger who always has an open ear for my concerns and who offered not only advice, but an incredible amount of support throughout all stages of this thesis. Maybe I could have done it without you, but I am so very glad that I did not have to try.

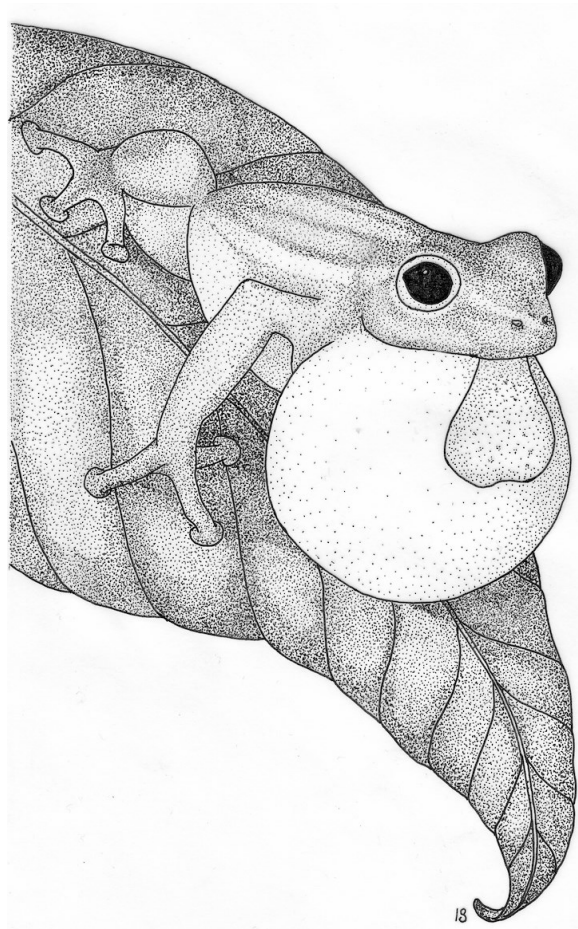
I would also like to thank Herbert Gasser for his support during the planning phase and especially for the great conversations we had during the final stages of both our theses. Conquering all bureaucratic challenges together and seeing you shine at your defence was what kept my motivation high during those last months.

Furthermore, I thank the Austrian Science Fund (FWF) for the financial support, the Tropical Biology Association (especially Clive Nuttman) for the tremendous help during 3 out of 4 field seasons, Max Dehling and Ulrich Sinsch (University of Koblenz) for their valuable advice and their support during the field season in Rwanda, Miguel Vences and Stefan Schulz (Technical University of Braunschweig) for the fruitful collaboration throughout my PhD studies, Manfred Walzl (University of Vienna) for his help with histological analyses of gular patch tissue and Klemens Bottig for providing specimens for analysis and for sharing his knowledge about reed frogs in captivity.

A big 'Thank you' goes to the administrative staff at the University of Vienna for walking the extra mile and making sure that everything usually works out. And although the network of colleagues is immensely important for a Phd-candidate to be successful, all of this would never be possible without the unconditional support of family and friends.

My sincere gratitude goes to my parents Elfi and Fritz, and to my sister Sonja for the continuous encouragement although you probably were quite worried when I announced that I wanted to collect data in East African swamps at night. I do hope that the happiness of me finally finishing my thesis helps you to get used to the strange title "Pi-äitsch-die".

And of course I would like to thank my partner Phil for being there for me on every step of the way. You often see my strength when I doubt it and encourage me to believe in myself.



Meinen Eltern Elfi und Fritz Starnberger

Table of Contents

Summary.....	1
Zusammenfassung.....	2
 Preface.....	 3
 Synopsis Of Publications.....	 9
Chapter	
1. Take time to smell the frogs: vocal sac glands of reed frogs (Anura: Hyperoliidae) contain species-specific chemical cocktails	11
2. From uni- to multimodality: towards an integrative view on anuran communication	25
3. The anuran vocal sac: a tool for multimodal signaling.....	39
4. Multimodal signal testing reveals gestural tapping display in the spotted reed frog	49
 Concluding Discussion	 81
 References	 87
 Appendix.....	 93
A. Multimodal signaling in the Small Torrent Frog (<i>Micrixalus saxicola</i>) in a complex acoustic environment	95
B. Take the long way home: Behaviour of a neotropical frog, <i>Allobates femoralis</i> , in a detour task	103
C. Identification, synthesis and mass spectrometry of a macrolide from the African reed frog <i>Hyperolius cinnamomeoventris</i>	109

Summary

The vocal sac present in males of most anuran species is generally associated with its role in advertisement call production. However, its function aside from acoustic communication was neglected for some time and cues such as vocal sac movement, were traditionally seen as an epiphenomenon of sound production. The diversity in vocal sac colouration and shape found in different species is striking and recently its visual properties were awarded an important role in signalling.

Males of many reed frog species (Anura: Hyperoliidae), have a prominent colourful patch on their vocal sac, which is very conspicuous once the vocal sac is inflated. The function of the gular patch makes up the central objective of this exploratory thesis.

I aim to highlight the unique hyperolid vocal sac and its potential function in multimodal signalling in particular, and the fascinating diversity and complexity of anuran multimodal signalling in general. To investigate the role of the vocal sac and the hyperolid gular patch in inter- and intra-specific communication, I integrated a literature research, histological and biochemical analyses in the laboratory as well as measurements of signal parameters and associated receiver response to signal components in cue-isolation experiments in the field.

I demonstrate that the gular patch is a gland complex producing species-specific chemical cocktails which are most likely expelled during calling - a so far undescribed mode of signalling in frogs. Furthermore, field experiments testing receiver-response to uni- and multimodal signal stimuli showed that the colourful gular patch does not act as a visual signal in male-male communication, but revealed a previously undescribed foot-tapping behaviour.

In two chapters of my thesis I sum up the current knowledge on uni- and multimodal signalling strategies in anurans and review the versatile role of the vocal sac in the anuran communication system.

Finally I discuss the mechanism and function of the vocal sac in acoustic, visual and chemical signalling of reed frogs to contribute to our understanding of the evolution of multimodal communication in the animal kingdom.

Zusammenfassung

Die Schallblase, die bei Männchen fast aller Anurenarten vorhanden ist, wurde meist im Zusammenhang mit der Produktion von Anzeigerufen untersucht. Die Funktion der Schallblase jenseits der akustischen Kommunikation wurde lange Zeit kaum beachtet und Attribute, wie etwa Schallblasenbewegung wurden traditionell eher als Epiphänomen der Ruferzeugung gesehen. Die Diversität von Schallblasenfarbe und -form bei verschiedenen Anurenarten ist beeindruckend und seit wenigen Jahren wird ihren optischen Eigenschaften und ihrer möglichen Rolle in der visuellen Signalgebung mehr Aufmerksamkeit zuteil.

Männliche Riedfrösche (Anura: Hyperoliidae) vieler Arten besitzen einen auffällig gefärbten Fleck auf der Schallblase, der besonders hervorsticht wenn die Schallblase mit Luft gefüllt ist. Die Funktion dieses Kehlflecks war gänzlich unbekannt und ist das zentrale Thema meiner explorativen Dissertation.

Ziel meiner Arbeit ist es, die einzigartige Riedfroschschallblase und ihre mögliche Rolle bei multimodaler Signalgebung, sowie ganz allgemein die faszinierende Diversität und Komplexität multimodaler Signale bei Anuren zu beleuchten. Um die Rolle der Schallblase und des Kehlflecks in inter- und intra-spezifischer Kommunikation besser zu verstehen, wurden histologische und biochemische Analysen im Labor durchgeführt sowie Messungen von Signalparametern und Empfängerreaktionen auf isolierte und kombinierte Signalkomponenten in Cue-Isolationsexperimenten im Freiland integriert.

Ich konnte zeigen, dass der Kehlfleck ein Drüsenkomplex ist, der artspezifische chemische Cocktails erzeugt, welche höchstwahrscheinlich während des Rufens ausgestoßen werden und somit einen bisher unbeschriebenen Signalmodus bei Anuren darstellen. Weiters stellte sich bei Feldversuchen, in denen die Reaktion von Empfängern auf uni- und multimodale Signale getestet wurde heraus, dass der farbige Kehlfleck keine Rolle in der Kommunikation zwischen Männchen spielt, aber ein bisher unbeschriebenes Verhalten gezeigt wird, bei dem mit dem Fuß auf den Untergrund geklopft wird.

In zwei Kapiteln meiner Arbeit, fasse ich den aktuellen Wissensstand zu uni- und multimodalen Signalstrategien bei Riedfröschen, sowie die vielfältige Rolle der Schallblase in der Kommunikation zusammen.

Abschließend diskutiere ich den Mechanismus und die Funktion der Schallblase in der akustischen, visuellen und chemischen Kommunikation von Riedfröschen, um zum Verständnis der Evolution der multimodalen Kommunikation im Tierreich beitragen zu können.

Preface

Communication in the Animal Kingdom

My fascination with **communication** strategies in the animal kingdom, **signal evolution** and **behavioural ecology** started during my undergraduate years, grew throughout my studies and finally led me to delve even deeper into the matter during my PhD studies.

"For communication to occur, a sender has to encode information in a signal, which is then transmitted to a receiver" (Shannon, 1948).

An individual's interaction with other living beings or at least with other conspecifics underpins the transmission of signals. The way it communicates can have detrimental effects on an individual's survival and evolutionary fitness, it is not surprising, that a myriad of different ways to send and receive information have developed through natural and sexual selection over evolutionary time (Bradbury & Vehrencamp, 2011; Dawkins & Guilford, 1997; Guilford & Dawkins, 1991). The vast **diversity of signalling strategies** naturally goes hand in hand with the diversity of **complex organs** to receive and decode information.

The often intricate ornaments which influence receivers and perceivers are those which also catch the eye of the scientifically inclined beholder. However, in order to understand how signals evolve, it is important to distinguish between a **signal**, a trait that evolved to trigger changes in the behaviour of receivers in the context of communication, and a **cue**, an informative trait that is not primarily selected for its communicative function, but rather acts as a guide for future actions (reviewed in Higham & Hebets, 2013). Setting out to answer the questions which the organism poses, can be enough to keep a **taxon-oriented scientist** curious and busy throughout his or her life (West-Eberhard, 2001). Thus, research on animal signalling typically connects at least Behavioural and Evolutionary Biology, as well as Physiology and Behavioural Ecology.

In several lectures I was introduced to different signal modalities: The striking song of a blackbird or a lion's roar are **acoustic** signals, which can typically travel over long distances (Wahlberg & Larsen, 2017). Acoustic signals can be very complex and therefore convey a multitude of information as in the case of human speech (reviewed by Fitch, 2000). However, acoustic signals typically quickly degrade and attenuate in the environment and can be rather costly to produce (e.g. Gaucher, 2002; Grafe & Thein, 2001) unlike the scent trails of the female silkworm moth (Saturnidae), a famous example of **chemical** signals (Butenandt, Beckmann, & Hecker, 1961). These signals and/or cues have the potential to inform receivers about a signaler's identity, reproductive state, and also the age of the scent mark, as shown not only for the Eurasian lynx (Vogt, Boos, Breitenmoser, & Kölliker, 2016). Metabolic cost for chemical signals

is usually low in comparison to other modalities (Wyatt, 2008) and their range can be quite broad (Ryan & Kime, 2003; Vane-Wright & Boppré, 1993).

Signals in the **visual** domain reach a medium to long range and can be very dynamic, such as the mimics of a domestic horse (Wathan, Proops, Grounds, & McComb, 2016) and the bioluminescent signals in fireflies (Lampyridae) (e.g. De Cock, Faust, & Lewis, 2014) or can be rather static but conspicuous under certain circumstances (e.g. light conditions, high contrast to immediate background), like the strikingly coloured fins of male guppies (*Poecilia reticulata*) (Endler, 1991).

We are also able to measure **seismic** signals, such as the infrasound rumbles of African elephants, which can cover long distances (reviewed by O'Connell-Rodwell, 2007) and crocodilians (Dinets, 2013) or the vibrations produced by male wolf spiders during courtship (Gibson & Uetz, 2007). For golden moles (*Eremitalpa granti*) substrate-borne low-frequency vibrations produced by conspecifics but also by desert grass patches are vital for orientation around their habitat (as reviewed by Mason & Narins, 2002).

Tactile signals which are usually transmitted directly from sender to receiver act on a very short range, but with a fast transmission rate and they facilitate the locatability of the sender even in complete darkness (Wyatt, 2008). Tactile communication is very common in eu-social species, such as honeybees (e.g. Lenoir & Jaisson, 1982), but also in social species, such as cows, whose reciprocal grooming behaviour has been shown to play an important role in bonding (Sato, Tarumizu, & Hatae, 1993).

A lesser known modality is **electric** signalling used for example by Elephant fishes (Mormyridae). Electric discharges originally used for electrolocation can be of advantage in intra-specific social communication as they provide a noise immune channel even in turbulent, murky waters (e.g. Hopkins, 1981).

Frogs are mostly known for their vocal communication; especially chorusing of many anuran species can be very impressive to human listeners. And acoustic signalling in frogs can reach extraordinary complexity, such as in the Chinese frog *Amolops tormotus*, whose advertisement calls are comparable to birdsong with many up- and downsweeps but also contain ultrasound frequency components (Feng, Narins, & Xu, 2002). The prominence of anuran acoustic signals has been reflected in scientific studies on frogs and toads over the last decades as beautifully illustrated in a meta study by Coleman (2009; and see Waldman & Bishop, 2004). However, there is increasing evidence that in many anuran species acoustic signals are only a part of the picture.

Multimodal Communication in Animals

In many species, not only one communication channel, but two or more are used **simultaneously** or **sequentially** across multiple sensory modalities (reviewed in Candolin, 2003; Hebets & Papaj, 2005) and in the last decade studies investigating multimodal communication systems in many taxa increased enormously (Farris & Taylor, 2017).

Complex multimodal signalling could not only be described, but systematically investigated in several invertebrate taxa, such as spiders where vibrational (seismic) signals are paired with dynamic visual signals in striking courtship displays (Hebets, 2008; Kozak & Uetz, 2014; Uetz, Roberts, Clark, Gibson, & Gordon, 2013) and butterflies (Papke, Kemp, & Rutowski, 2007), to name just two examples of many.

There is increasing evidence that multimodal signalling is much more elaborate and much more common in amphibians in general and in anurans in particular than previously suspected (Amézquita & Hödl, 2004; Narins, 1990; Narins, Hödl, & Grabul, 2003; Preininger, Boeckle, & Hödl, 2009). An advantage of multimodal communication might be an **enhanced signal to noise ratio** (Gerhardt & Schwartz, 2001) and it could provide reliable indicators to attributes of a sender (Gomez et al., 2009; Richardson et al., 2010; e.g. Vásquez & Pfennig, 2007). Narins et al. (2003; 2005) demonstrated that in the poison dart frog *Allobates femoralis* simultaneous acoustic and visual signals are necessary to evoke an aggressive reaction in a male defending a territory. Taylor et al. (2007) found that female squirrel tree frogs (*Hyla squirella*) prefer a male model frog presented simultaneously with a male call over the male call only.

Although it seems that in all anurans investigated so far there is a general preference for multimodal signals, vast differences can be seen in the importance of signal components and responses they elicit in the receiver even in species facing similar ecological problems (Taylor, Klein, & Michael, 2011). An essential requirement to understand multimodal signal influence is to perform experiments which break down a multimodal signal into its **components** and to analyze the associated **receiver responses** to isolated and combined components (e.g. Hebets & Papaj, 2005; Taylor et al., 2011). This would not only enable the comparison with other species but also the analysis of signal function.

This study aims to investigate for the first time the function of a possible **trimodal (acoustic, visual and chemical) communication** system associated with the vocal sac in reed frogs by means of **interdisciplinary methods** and by testing the receiver's reaction to single and combined signal components.

Structure and Content of this Thesis

Males of the genus *Hyperolius* call in a multi-species chorus at night sitting on plant structures next to or in an aquatic site. Their large and colourful vocal sac with one central gular pad is very conspicuous to the human observer, however, little was known about the **function of the gular patch**. 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 numbers, locations and shapes of gular patches in reed frogs and described them as glands whose „actual function remains obscure“. Some genera have gular patches but lack a vocal sac and Rödel et al. (2003) suggest chemical communication in two apparently mute hyperoliid species from West Africa in which males have two gland-like structures on the throat.

Fascinating observations in the field and a taxon-centered approach (West-Eberhard, 2001) led to this explorative thesis in which I aim to contribute to our understanding of the complex multimodal signalling system focusing on the vocal sac as a "signalling hub" in reed frogs.

To understand how certain signal components are produced and to investigate their intra- and interspecific evolutionary function in the communication system of reed frogs, I investigated uni- and multimodal signals in the acoustic, visual and chemical domain. The crucial questions guiding my scientific work were:

A) What is the mechanism and function of the gular patch of reed frogs?

B) Which role does multimodal signalling play in anurans?

C) Which role does the vocal sac play in anuran communication?

To tackle these questions, I used a diverse set of methods ranging from literature research and histological analyses to acoustic recordings and behavioral experiments in the field.

In **Chapter 1** I present results which were obtained in the lab and in the field. Together with my collaborators, I investigated the internal structure of the hyperolid gular patch in four sympatric species as well as the chemical scent bouquets potentially emitted during calling.

Chapters 2 and 3 consist of invited review articles in which I sum up the current knowledge on uni- and multimodal signalling strategies in anurans.

The results of **Chapter 4** allow first insights into the multimodal signalling system of a reed frog species. Presenting *Hyperolius puncticulatus* with artificial vocal sacs in field experiments yielded surprising results in regard to acoustic, visual and potential seismic communication.

Synopsis of Publications

Chapter 1

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

Starnberger I., Poth D., Saradhi Peram P., Schulz S., Vences M., Knudsen J., Barej M. F., Rödel M.-O., Walzl M. & W. Hödl

Publication released: Biological Journal of the Linnean Society 110(4): 828-838. DOI: 10.1111/bij.12167

Contribution: **IS**, SS, MV, JK, MW, WH designed the experiments. **IS**, JK, WH performed the experiments in the field and collected samples. **IS**, DP, PSP, SS, MV, JK, MFB, M.-O.R analyzed the data. MV, MFB contributed samples. MW, **IS** performed histological analyses. **IS**, DP, MV, M.-O.R wrote the paper.

Chapter 2

Invited Review: From uni- to multimodality: towards an integrative view on anuran communication

Starnberger I., Preininger D., & W. Hödl. 2014

Publication released: Journal of Comparative Physiology A 200:777–787. DOI:10.1007/s00359-014-0923-1

Contribution: **IS**, DP, WH designed the composition and performed literature research. **IS**, DP wrote the paper.

Chapter 3

Invited Review: The anuran vocal sac: a tool for multimodal signaling

Starnberger I., Preininger D., & W. Hödl. 2014

Publication released: Animal Behaviour: 97: 281-288, Special Issue: Biochemistry & Animal Communication. DOI: 10.1016/j.anbehav.2014.07.027

Contribution: **IS**, DP, WH designed the composition and performed literature research. **IS**, DP wrote the paper.

Chapter 4

Research Paper: Multimodal signal testing reveals gestural tapping behavior in the Spotted Reed Frog

Starnberger I., Maier P.M., Hödl W. & D. Preininger

Manuscript accepted: 2018-02-02 Herpetologica

Contribution: **IS**, DP, WH designed the experiments. **IS**, PMM performed the experiments. **IS**, DP: Analyzed the data. **IS**, DP wrote the paper.

Chapter 1

TAKE TIME TO SMELL THE FROGS:

VOCAL SAC GLANDS OF REED FROGS (ANURA: HYPEROLIIDAE) CONTAIN SPECIES-SPECIFIC CHEMICAL COCKTAILS

IRIS STARNBERGER, DENNIS POTH, PARDHA S. PERAM, STEFAN SCHULZ, MIGUEL VENCES, JETTE KNUDSEN, MICHAEL F. BAREJ, MARK-OLIVER RÖDEL, MANFRED WALZL AND WALTER HÖDL



Biological Journal of the Linnean Society, 110(4), 828-838. doi: 10.1111/bij.12167

Received 21 May 2013; Accepted 26 July 2013; Published 1 December 2013

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

IRIS STARNBERGER^{1*}, DENNIS POTH², PARDHA SARADHI PERAM², STEFAN SCHULZ², MIGUEL VENCES³, JETTE KNUDSEN⁴, MICHAEL F. BAREJ⁵, MARK-OLIVER RÖDEL⁵, MANFRED WALZL¹ and WALTER HÖDL¹

¹Department of Integrative Zoology, University of Vienna, Althanstrasse 14, Wien 1090, Austria

²Institute for Organic Chemistry, Technical University of Braunschweig, Braunschweig, Germany

³Zoological Institute, Technical University of Braunschweig, Braunschweig, Germany

⁴Department of Biology, Lund University, Lund, Sweden

⁵Museum für Naturkunde Berlin, Leibniz Institute for Research on Evolution and Biodiversity, Berlin, Germany

Received 21 May 2013; revised 26 July 2013; accepted for publication 26 July 2013

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 *Afraxalus*, *Heterixalus*, *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.

ADDITIONAL KEYWORDS: *Afraxalus* – Amphibia – chemical communication – *Heterixalus* – histology – *Hyperolius* – gular gland anatomy – pheromones – *Phlyctimantis*.

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 Hdl & Amzquita (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 & Hdl (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; Rdel *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. H. dl, 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). R del *et al.* (2003) provided further evidence for possible chemical communication in two apparently mute hyperoliid species from West and Central Africa [*Acanthixalus sonjae* R del, 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⁻¹ 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

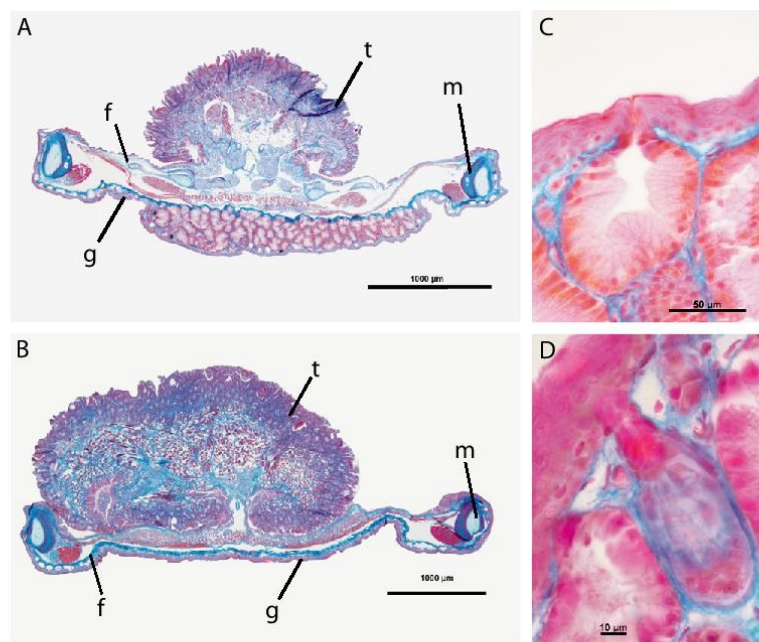


Figure 2. Cross sections (6-µm thick; AZAN stain) of the lower jaw of a male (A) and a female (B) *Hyperolius viridiflavus*, showing the tongue (t), the mandibular arch (m), the floor of the mouth (f), and the gular skin (g). The male's gular skin is extremely hypertrophied and forms a tubular gland 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.

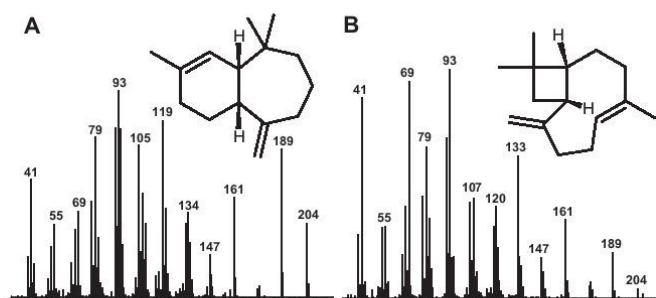
sac tissue (Fig. 2A, C and D). The gular patch is situated in the stratum spongiosum within the dermis, and comprises three different gland types. We hereafter use the term 'gular gland' to refer to this macrogland structure. A vast part of the tissue is made up of glands consisting of densely packed, 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 (*Afraxalus* 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 *Afraxalus lacteus* Perret, 1976 (one compound). Furthermore, we found a small number of macrolides in two *Hyperolius* species and in *Afraxalus 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>Afraxalus</i>	<i>lacteus</i>	3	1	1			5
<i>Afraxalus</i>	<i>laevis</i>	3	2				5
<i>Afraxalus</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* (α -himachalene, A) and from *Hyperolius kivuensis* [2-epi-(*E*)- β -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 α -himachalene and 2-epi-(*E*)- β -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

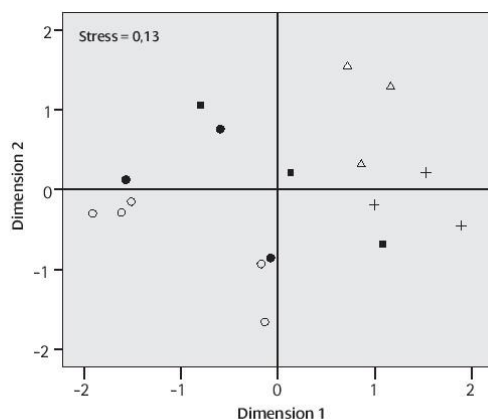


Figure 4. Non-metric multidimensional scaling (nMDS) plot of *Hyperolius* chemical compound profiles among four sympatric species. The nMDS plot is based on the presence/absence of 65 compounds in the gular patch tissue samples (Table S1). Each symbol represents an individual frog. Symbol shape refers to species identity (Δ , *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. R. del, 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*, *Afraxalus*, 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. L. tters *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. H dl (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 (R del, 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 & H dl, 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.

ACKNOWLEDGEMENTS

We thank A. Am zquita, N. L. Gonwouo, M. Hirschfeld, A. Lembens, H. C. Liedtke, and I. Maiditsch for their help in the field, and F. and M. Kamoga and the Tropical Biology Association for helping us with logistics and the acquisition of permits. D. Preininger and M. Sztatecsny helped with equipment and valuable advice. Our study received financial support from the Austrian Science Fund (FWF): W1234 and P25612, a seed grant of the Zukunftsfonds (Programmpauschale) of TU

Braunschweig, the Deutsche Forschungsgemeinschaft (grant no. Schu984/10-1), and was co-financed by a research grant of the University of Vienna, 2011. Specimen collection and export was authorized by the Uganda Wildlife Authority, the Uganda National Council for Science and Technology (permit NS 374). Specimen collection in Cameroon was conducted within the research project 0603/P/MINFOR/SG approved by the Cameroonian Ministries MINFOR and MINRESI. The collection of samples of *Heterixalus* in Madagascar was carried out in the framework of a collaboration between T.U. Braunschweig and the Universit d'Antananarivo. We thank D.C. Blackburn and three anonymous reviewers for their constructive criticism and their encouraging comments.

REFERENCES

- Adams RP. 1995. *Identification of essential oil components by gas chromatography-mass spectrometry*. Carol Stream: Allured Publishing Corporation.
- Amiet JL. 1972. Notes faunistiques, biologiques etologiques sur quelques amphibiens anoures du Cameroun. *Annals de la Faculté des Sciences du Cameroun* 9: 127–153.
- Amiet JL. 2012. Les rainettes du Cameroun. Nyons & Saint Nazaire: Edition J.-L. Amiet & La Nef des Livres.
- Bee MA, Micheyl C. 2008. The cocktail party problem: what is it? How can it be solved? And why should animal behaviorists study it? *Journal of Comparative Psychology* 122: 235–251.
- Belanger R, Corkum L. 2009. Review of aquatic sex pheromones and chemical communication in anurans. *Journal of Herpetology* 43: 184–191.
- Boeckle M, Preininger D, H dl W. 2009. Communication in noisy environments I: acoustic signals of *Staurois latopalmatus* Boulenger 1887. *Herpetologica* 65: 154–165.
- Bucher TL, Ryan MJ, Bartholomew GA. 1982. Oxygen-consumption during resting, calling, and nest building in the frog *Physalaemus pustulosus*. *Physiological Zoology* 55: 10–22.
- Byrne P, Keogh J. 2007. Terrestrial toadlets use chemosignals to recognize conspecifics, locate mates and strategically adjust calling behaviour. *Animal Behaviour* 74: 1155–1162.
- Caldwell MS, Johnston GR, McDaniel JG, Warkentin KM. 2010. Vibrational signaling in the agonistic interactions of red-eyed treefrogs. *Current Biology* 20: 1012–1017.
- Carson FL. 1997. *Histotechnology: a self-instructional text*. Chicago: American Society for Clinical Pathology.
- Coleman S. 2009. Taxonomic and sensory biases in the mate-choice literature: there are far too few studies of chemical and multimodal communication. *Acta Ethologica* 12: 45–48.
- Dorcas ME, Price SJ, Walls SC, Barichivich WJ. 2010. Amphibian ecology and conservation: a handbook of techniques. In: Dodd KC, ed. *Auditory monitoring of anuran populations*. Oxford: Oxford University Press, 281–298.
- Drewes R. 1984. A phylogenetic analysis of the Hyperoliidae (Anura): treefrogs of Africa, Madagascar, and the Seychelles Islands. *Occasional Papers of the California Academy of Sciences* 139: 1–70.
- Duellman WE, Trueb L. 1986. *Biology of Amphibians*. New York: McGraw-Hill Publishing Company.
- Endler JA, Thery M. 1996. Interacting effects of lek placement, display behavior, ambient light, and color patterns in three Neotropical forest-dwelling birds. *American Naturalist* 148: 421–452.
- Fleishman L, McClintock WJ, D'Eath R, Brainard D, Endler JA. 1998. Colour perception and the use of video playback experiments in animal behaviour. *Animal Behaviour* 56: 1035–1040.
- Flowers MA, Graves BM. 1997. Juvenile toads avoid chemical cues from snake predators. *Animal Behaviour* 53: 641–646.
- Forester DC, Thompson KJ. 1998. Gauntlet behaviour as a male sexual tactic in the American toad (Amphibia: Bufonidae). *Behaviour* 135: 99–119.
- Frost DR. 2012. Amphibian Species of the World: an online reference. Version 5.6 (1 October 2012). Electronic Database accessible at <http://research.amnh.org/herpetology/amphibia/index.html>. American Museum of Natural History, New York, USA.
- Gerhardt H, Huber F. 2002. *Acoustic communication in insects and anurans: common problems and diverse solutions*. Chicago: University of Chicago Press.
- Hamer R, Lemckert FL, Banks PB. 2011. Adult frogs are sensitive to the predation risks of olfactory communication. *Biology Letters* 7: 361–363.
- Hamlett BE, Strecker AG, Trauth SE. 1998. Caudal courtship glands in the cave salamander, *Eurycea lucifuga* (Caudata: Plethodontidae). *Journal of the Arkansas Academy of Science* 52: 124–128.
- Hammer O, Harper DAT, Ryan PD. 2001. PAST: paleontological Statistics software package for education and data analysis. *Paleontologia Electronica* 4: 9.
- Hedin PA, Maxwell FG, Jenkins JN. 1974. Insect plant attractants, feeding stimulants, repellents, deterrents and other related factors affecting insect behavior. In: Maxwell FG, Harris FA, eds. *Proceedings, summer institute on biological control of plants and diseases*. Jackson: Mississippi University Press, 494–527.
- Hilton WA. 1902. A structural feature connected with the mating of *Dienyctulus viridescens*. *American Naturalist* 34: 643–651.
- Hirschmann W, H dl W. 2006. Visual signaling in *Phrynobatrachus krefftii* Boulenger, 1909 (Anura: Ranidae). *Herpetologica* 62: 18–27.
- H dl W. 1977. Call differences and calling site segregation in anuran species from Central Amazonian floating meadows. *Oecologia* 28: 351–363.
- H dl W, Am zquita A. 2001. Visual signaling in anuran amphibians. In: Ryan MJ, ed. *Anuran communication*. Washington: Smithsonian Institution Press, 121–141.
- Houck L. 2009. Pheromone communication in amphibians and reptiles. *Annual Review of Physiology* 71: 161–176.

- Johansson BG, Jones TM. 2007. The role of chemical communication in mate choice. *Biological Reviews* **82**: 265–289.
- Kime N, Turner W, Ryan M. 2000. The transmission of advertisement calls in Central American frogs. *Behavioral Ecology* **11**: 71–83.
- Korbeck RG, McRobert SP. 2005. Home area recognition via olfactory cues in the tropical poison frog *Dendrobates auratus*. *Russian Journal of Herpetology* **12**: 161–166.
- Lenzi-Mattos R, Antoniazzi M, Haddad C, Tambourgi D, Rodrigues M, Jared C. 2005. The inguinal macroglands of the frog *Physalaemus nattereri* (Leptodactylidae): structure, toxic secretion and relationship with deimatic behaviour. *Journal of Zoology* **266**: 385–394.
- Lewis E, Narins P, Cortopassi K, Yamada W, Poinar E, Moore W, Yu X. 2001. Do male white-lipped frogs use seismic signals for intraspecific communication? *Integrative and Comparative Biology* **41**: 1185–1193.
- Lutters S, Schick S, Scheelke K, Teege P, Kosuch J, Rotich D, Veith M. 2004. Bio-sketches and partitioning of sympatric reed frogs, genus *Hyperolius* (Amphibia; Hyperoliidae), in two humid tropical African forest regions. *Journal of Natural History* **38**: 1969–1997.
- Malacarne G, Giacoma C. 1986. Chemical signals in European newt courtship. *Italian Journal of Zoology* **53**: 79–83.
- Martins IA, Almeida SC, Jim J. 2006. Calling sites and acoustic partitioning in species of the *Hyla nana* and *rubicundula* groups (Anura, Hylidae). *The Herpetological Journal* **16**: 239–247.
- Partan S, Marler P. 2005. Issues in the classification of multimodal communication signals. *The American Naturalist* **166**: 231–245.
- Pauly GB, Bernal XE, Rand AS, Ryan M. 2006. The vocal sac increases call rate in the t ngara frog *Physalaemus pustulosus*. *Physiological and Biochemical Zoology* **79**: 708–719.
- Pearl C, Cervantes M, Chan M, Ho U, Shoji R, Thomas E. 2000. Evidence for a mate-attracting chemosignal in the dwarf African clawed frog *Hymenochirus*. *Hormones and Behavior* **38**: 67–74.
- Perret JL. 1961. La biologie d'*Acanthixalus spinosus* (Amphibia Salientia). *Recherches et Etudes camerounaises* **1**: 90–101.
- Poth D, Peram PS, Vences M, Schulz S. 2013. Macrolides and alcohols as scent gland constituents of the Madagascan frog *Mantidactylus femoralis* and their intraspecific diversity. *Journal of Natural Products* **76**: 1548–1558.
- Poth D, Wollenberg KC, Vences M, Schulz S. 2012. Volatile amphibian pheromones: macrolides from mantellid frogs from Madagascar. *Angewandte Chemie International Edition* **51**: 2187–2190.
- Rajchard J. 2005. Sex pheromones in amphibians: a review. *Veterinary Medicine-Czech* **50**: 385–389.
- Rand A, Dudley R. 1993. Frogs in helium: the anuran vocal sac is not a cavity resonator. *Physiological Zoology* **66**: 793–806.
- Richardson C, Gomez D, Durieux R, Thery M, Joly P, L na J, Plenet S, Lengagne T. 2010. Hearing is not necessarily believing in nocturnal anurans. *Biology Letters* **6**: 633–635.
- R del MO, Kosuch J, Veith M, Ernst R. 2003. First record of the genus *Acanthixalus* Laurent, 1944 from the Upper Guinean rain forest, West Africa, with the description of a new species. *Journal of Herpetology* **37**: 43–52.
- R del MO, Lampert KP, Linsenmair KE. 2006. Reproductive biology of the West African savannah frog *Hyperolius nasutus* G nther, 1864. *Herpetozoa* **19**: 3–12.
- Rosenthal G, Rand A, Ryan M. 2004. The vocal sac as a visual cue in anuran communication: an experimental analysis using video playback. *Animal Behaviour* **68**: 55–58.
- Russo T, Pulcini D, O'Leary , Cataudella S, Mariani S. 2008. Relationship between body shape and trophic niche segregation in two closely related sympatric fishes. *Journal of Fish Biology* **73**: 809–828.
- Ryan MJ. 1985. *The T ngara frog: a study in sexual selection and communication*. Chicago: University of Chicago Press.
- Schiötz A. 1967. The treefrogs (Rhacophoridae) of West Africa. *Spolia Zoologica Musei Hauniensis* **25**: 1–346.
- Schiötz A. 1999. *Treefrogs of Africa*. Frankfurt am Main: Edition Chimaira.
- Schmitz A, Euskirchen O, B hme W. 1999. Zur Herpetofauna einer montanen Regenwaldregion in SW-Kamerun (Mt. Kupe und Bakossi-Bergland). *herpetofauna* **21**: 5–17.
- Schulte LM, Yeager J, Schulte R, Veith M, Werner P, Beck LA, L tters S. 2011. The smell of success: choice of larval rearing sites by means of chemical cues in a Peruvian poison frog. *Animal Behaviour* **81**: 1147–1154.
- Sever DM. 1989. Caudal hedonic glands in salamanders of the *Eurycea bislineata* complex (Amphibia, Plethodontidae). *Herpetologica* **45**: 322–329.
- Sinsch U. 1990. Migration and orientation in anuran amphibians. *Ethology Ecology & Evolution* **2**: 65–79.
- Sinsch U, L mkemann K, Rosar K, Schwarz C, Dehling M. 2012. Acoustic niche partitioning in an anuran community inhabiting an Afromontane wetland (Butare, Rwanda). *African Zoology* **47**: 60–73.
- Taylor R, Buchanan B, Doherty J. 2007. Sexual selection in the squirrel treefrog *Hyla squirella*: the role of multimodal cue assessment in female choice. *Animal Behaviour* **74**: 1753–1763.
- Taylor R, Klein B, Stein J, Ryan M. 2008. Faux frogs: multimodal signalling and the value of robotics in animal behaviour. *Animal Behaviour* **76**: 1089–1097.
- Thomas EO, Tsang L, Licht P. 1993. Comparative histochemistry of the sexually dimorphic skin glands of anuran amphibians. *Copeia* **1993**: 133–143.
- Treer D, Van Bocxlaer I, Matthijs S, Four Du D, Janssenswillen S, Willaert B, Bossuyt F. 2013. Love is blind: indiscriminate female mating responses to male courtship pheromones in newts (Salamandridae). *PLoS ONE* **8**: e56538.
- Vences M, Kosuch J, Glaw F, B hme W, Veith M. 2003. Molecular phylogeny of hyperoliid treefrogs: biogeographic origin of Malagasy and Seychellean taxa and re-analysis of

- familial paraphyly. *Journal of Zoological Systematics and Evolutionary Research* **41**: 205–215.
- Vences M, Wahl-Boos G, Hoegg S, Glaw F, Spinelli Oliveira E, Meyer A, Perry S. 2007.** Molecular systematics of mantelline frogs from Madagascar and the evolution of their femoral glands. *Biological Journal of the Linnean Society* **92**: 529–539.
- Wabnitz P, Bowie JH, Tyler M, Wallace J, Smith B. 1999.** Animal behaviour: aquatic sex pheromone from a male tree frog. *Nature* **401**: 444–445.
- Waldman B, Bishop PJ. 2004.** Chemical communication in an archaic anuran amphibian. *Behavioral Ecology* **15**: 88–93.
- Wells KD, Schwartz J. 1982.** The effect of vegetation on the propagation of calls in the neotropical frog *Centrolenella fleischmanni*. *Herpetologica* **38**: 449–455.
- Wilbur HM, Rubenstein DI, Fairchild L. 1978.** Sexual selection in toads: the roles of female choice and male body size. *Evolution* **32**: 264–270.
- Woodley S. 2010.** Pheromonal communication in amphibians. *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology* **196**: 713–727.
- Zahavi A. 1975.** Mate selection: a selection for a handicap. *Journal of Theoretical Biology* **53**: 205–214.

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.

Chapter 2

**INVITED REVIEW:
FROM UNI- TO MULTIMODALITY:
TOWARDS AN INTEGRATIVE VIEW ON ANURAN COMMUNICATION**

IRIS STARNBERGER, DORIS PREININGER AND WALTER HÖDL



Journal of Comparative Physiology A 200:777–787. DOI:10.1007/s00359-014-0923-1

Received 25 March 2014; Accepted 8 June 2014; Published 29 June 2014

From uni- to multimodality: towards an integrative view on anuran communication

Iris Starnberger · Doris Preininger · Walter H. dl

Received: 25 March 2014 / Revised: 6 June 2014 / Accepted: 8 June 2014
© The Author(s) 2014. This article is published with open access at Springerlink.com

Abstract Undeniably, acoustic signals are the predominant mode of communication in frogs and toads. Acoustically active species are found throughout the vast diversity of anuran families. However, additional or alternative signal modalities have gained increasing attention. In several anurans, seismic, visual and chemical communications have convergently evolved due to ecological constraints such as noisy environments. The production of a visual cue, like the inevitably moving vocal sac of acoustically advertising males, is emphasized by conspicuously coloured throats. Limb movements accompanied by dynamic displays of bright colours are additional examples of striking visual signals independent of vocalizations. In some multimodal anuran communication systems, the acoustic component acts as an alert signal, which alters the receiver attention to the following visual display. Recent findings of colourful glands on vocal sacs, producing volatile species-specific scent bouquets suggest the possibility of integration of acoustic, visual and chemical cues in species recognition and mate choice. The combination of signal components facilitates a broadened display repertoire in challenging environmental conditions. Thus, the complexity of the communication systems of frogs and toads may have been underestimated.

Keywords Bioacoustics · Visual signals · Chemical signals · Frogs · Toads · Signal modalities

I. Starnberger (✉) · D. Preininger · W. H. dl
Department of Integrative Zoology, University of Vienna,
Althanstraße 14, 1090 Vienna, Austria
e-mail: iris.starnberger@univie.ac.at

D. Preininger
Vienna Zoo, Maxingstraße 13b, 1130 Vienna, Austria

Introduction

A great diversity of signalling strategies and behaviours can be observed during animal communication shaped by sexual selection and the environmental constraints (Narins and Zelick 1988; Endler 1992; Endler and Thery 1996; Leal and Fleishman 2004; Bradbury and Vehrencamp 2011). In several species, not only one communication mode, but two or more are used simultaneously or sequentially across multiple sensory components (Partan and Marler 1999; reviewed in Candolin 2003; Hebets and Papaj 2005; Otovic and Partan 2009). Multimodal communication is discussed for a wide range of species including spiders (e.g. Uetz et al. 2009), fish (e.g. Van Staaden and Smith 2011), reptiles (reviewed in Hews and Martins 2013), birds (e.g. Wiley 1973) and mammals (e.g. Bro-Jorgensen and Dabelsteen 2008), but has been difficult to test until recently. Complex signalling repertoires are challenging to investigate and valid hypotheses testing remains difficult in scientific experiments (Leger 1993; Partan and Marler 2005; Rosenthal 2007). However, advances in conceptual framework and technical equipment have greatly improved research in this field. In anuran amphibians, calls are the predominant signals in inter- and intrasexual communication (Ryan 1985; Gerhardt and Huber 2002; Dorcas et al. 2010). Vocalizations are the most conspicuous display to human observers and as a consequence other signal modalities have traditionally received less attention (Waldman and Bishop 2004; Coleman 2009) or may have been misjudged, e.g. due to experimenters' lack of visual sensitivity at night (Buchanan 1993). Amphibians in general and frogs in particular are excellent model organisms to experimentally investigate communication strategies both in the laboratory and under natural conditions, as they are hardly disturbed by observers and can be easily

manipulated (Narins et al. 2003; Hirschmann and H dl 2006; Taylor et al. 2007).

This review highlights anuran signalling strategies in addition to calling to promote an integrative multimodal view on anuran communication.

Anurans are born to call

“Frogs enjoy life and express their joy by song” (Dickerson 1908). Much has changed since these lines were written, and due to numerous studies on acoustic signals in anurans, we have a very different albeit less romantic opinion on signal content and function. The male advertisement call attracts conspecific females and signals the readiness to defend territories and calling sites to rival males; hence calling behaviour plays a vital role in reproductive success and is essential for sexual selection (Narins et al. 2007). Many frogs and toads have more than one species-specific call type. In addition to the prominent advertisement call a variety of discrete or continuous call types correspond to specific functions, such as the encounter call (McDiarmid and Adler 1974), the courtship call, the territorial call, the distress call, and the release call (all reviewed in Wells 1977).

How frogs and toads get their acoustic message across

In numerous species, audio-spectral and temporal call characteristics and their function as static or dynamic signal properties were investigated during the last decades (Gerhardt and Huber 2002). Robert R. Capranica was the first to combine electrophysiological analyses, behavioural data and synthetic playback calls to study “what the frog’s ear tells the frog’s brain” (Capranica and Moffat 1983; and see Simmons 2012). As a mentor, he inspired future generations to study animal communication by means of integrative research in the lab and also in the field and opened-up a new field which likewise attracted bioacousticians and evolutionary biologists. As a consequence, anuran vocal signals and their perception are nowadays an exceptionally well-understood subject in biology. Anuran call characteristics correlate with body size and mass across species (Ryan 1988; Gingras et al. 2013) and within species (e.g. Narins and Smith 1986; Robertson 1990) a pattern described as the Deep croak hypothesis by Davies and Halliday (1979); and see Gingras et al. (2013). Furthermore, call parameters signal species identity (e.g. Blair 1958; H dl 1977), and in some species exhibit “individual” distinctive signatures (Bee and Gerhardt 2001a, b; Gasser et al. 2009), but not in others (Bee 2003). Advertisement calls also regulate male spacing (e.g. Brenowitz 1989), increase the male’s attractiveness to females (e.g. Ryan and Keddy-Hector 1992) and

have evolved to match the tuning of the receiver’s auditory system (Gerhardt and Schwarz 2001).

Any message needs to be successfully transmitted to elicit the intended response in the receiver (Shannon 1948); clear reception is a minimum requirement for a successful communication system (Shannon 1948; Endler 1993). The most basic requirement for a call is to be detectable against background noise and to minimize transmission degradation and attenuation of the environment. High levels of biotic and abiotic environmental noise may mask calls and hamper accurate detection, discrimination and localization (or increase response latency) by receivers (Bee 2008; V lez et al. 2012, 2013; Caldwell and Bee 2014). However, signal properties and strategies have been shaped over evolutionary time to enhance transmission in their respective acoustic environments by preferences of receivers.

To improve signalling effectiveness, anuran species organize calling periods temporally (Klump and Gerhardt 1992), adjust their calls to random intercall intervals (Zelick and Narins 1985) or inhibit calling heterospecifics (Schwartz and Wells 1983). In dense breeding aggregations, concurrently chorusing conspecifics can achieve release from masking interference by spatial separation from the biotic sound source (Grafe 1996; Bee 2008). In the presence of continuous background noise, the recognition and detection of acoustic signals can be impaired dependent on the relative noise level and frequency (Feng and Schul 2006). In torrent frogs, high-frequency and in some cases even ultrasonic calls enhance the signal-to-noise ratio relative to low-frequency stream noise and are suggested to be an adaptive strategy in the presence of continuous noise (Narins et al. 2004; Feng et al. 2006; Boeckle et al. 2009). For example, stream-associated species of the genus *Staurois* emit higher pitched calls than other ranid species of comparable body size (Fig. 1).

Acoustic adaptations to overcome signalling constraints are limited and often opposed by morphological and phylogenetic constraints—but most importantly by sexual selection (e.g. Ryan and Rand 2003). In many cases, species develop new, often spectacular ways of communication in which the production of acoustic signals is of less importance or even completely abandoned (R del et al. 2003; Hirschmann and H dl 2006; Preininger et al. 2009).

When calling is not enough: from single to multimodality

“Compared to salamanders, olfactory cues and visual displays seem to be unimportant to preamplectic courtship in most anurans, but some tactile cues are used by certain species.” Duellman and Trueb (1986) wrote in their widely used textbook “Biology of Amphibians” published almost

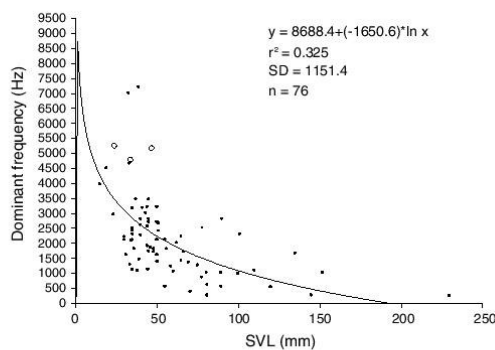


Fig. 1 Logarithmic regression of call frequency on body size of 76 male ranid frogs. Circles denote foot-flagging species of the stream-associated genus *Staurois* (*S. parvus*, left; *S. guttatus*, middle; *S. latopalmatus*, right) emitting higher pitched calls than other ranids of similar body size. Graph was adapted with permission of the authors, for detailed data on the species see (Boeckle et al. 2009)

30 years ago. Today numerous new findings in several anuran species in regard to colourations, visual displays and even olfactory cues utilized as signals in conspecific communication depict a somewhat different story.

The role of the vocal sac in communication

The anuran vocal sac likely evolved in response to selection for increased calling efficiency (Bucher et al. 1982; Pauly et al. 2006). 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 et al. 1982; Rand and Dudley 1993; Pauly et al. 2006). Colour and shape of the vocal sac display were probably first incorporated as a visual cue and selection pressures later shaped various conspicuous signal variations. The visual component of the vocal sac increases detection through movement and colouration (Rosenthal et al. 2004; Taylor et al. 2008), thereby enhancing the call attractiveness to females and aggression during territorial male–male interactions. Females of the T ngara frog (*Engystomops pustulosus*) prefer advertisement calls in addition to the visual cue of a pulsating vocal sac over the call alone under low sound pressure levels. However, when the visual stimulus is presented with a less attractive slow call rate, females rather choose the attractive unimodal call, which emphasizes that vocalizations are necessary for mate attraction (Taylor et al. 2011a). Comparisons of unimodal acoustic stimuli to multimodal stimuli presentations in the Kottigehar Dancing Frog (*Micrixalus kottigeharensis*; former identification as Small Torrent Frog (*Micrixalus* aff. *saxicola*))



Fig. 2 A rendering from video frame illustrating aggressive (fighting) behaviour of an *A. femoralis* male (right) toward the electromechanical model frog (left) placed in his territory, 2 m from his initial calling position. In all experimental trials that evoked fighting, the model's vocal sac was inflated and pulsating, and was accompanied by playback of the male's species-specific territorial call. With kind permission: Narins et al. (2003), Fig. 3. Copyright (2003) National Academy of Sciences, USA

demonstrated that a pulsating white vocal sac increases the frequency of response behaviours in conspecific males and elicits an agonistic visual display response (Preininger et al. 2013c). The visual cue of the vocal sac was suggested to mainly facilitate detection and localization of mating partners or opponents in noisy choruses (Rosenthal et al. 2004; Taylor et al. 2008, 2011b; Preininger et al. 2013a).

The first evidence for the role of a pulsating vocal sac as a visual cue during male territorial defense comes from studies in the brilliant thighed dart-poison frog *Allobates femoralis*. No unimodal stimulus (call or pulsating vocal sac) was able to elicit territorial aggression in the opponent male, only temporally overlapping dynamic bimodal cues evoked fighting behaviour (Narins et al. 2003, 2005). Hence, conspecific vocalizations in *A. femoralis* trigger a phonotactic response and antiphonal calling but are not sufficient to evoke physical aggression (Fig. 2). De Luna et al. (2010) showed that the movement of the inflated grey vocal sac itself was not important, but movement of the frog dummy per se (i.e. jumping) evoked territorial aggression in *A. femoralis* males. Conspicuous colourations, however, are often crucial in visual vocal sac displays, such as in the East-African stream frog *Phrynobatrachus krefftii* where conspicuous yellow vocal sacs function as signals in male–male agonistic interactions even without calls being emitted (Hirschmann and Hölzl 2006).

The inevitable movement of the vocal sac during sound production can also act as vibrational or seismic cue. In the mostly ground-dwelling white-lipped frog *Leptodactylus*

albilabris vocal sac inflations against the ground produce substrate-borne vibrations (Lewis et al. 2001) which act as additional seismic signals to overcome heterospecific chorus noise (and see Cardoso and Heyer 1995). Frogs and toads calling in the water produce circular waves travelling from the calling individual. It has been shown in the lateral line bearing Fire-bellied toad *Bombina bombina*, that individuals floating in the vicinity of the caller perceive this water movement and respond to it (Seidel et al. 2001). When males of the nocturnal Red-eyed tree frog (*Agalychnis callidryas*) perceive plant-borne vibrations indicating a conspecific intruder, they in turn shake branches as an agonistic display (Caldwell et al. 2010). The tremulations of *A. callidryas* correlate with individual dominance and might play a role in sexual selection, comparable to seismic signals in wolf spiders (Elias et al. 2005), moreover, they constitute a signalling modality dissociated from vocalizations.

Visual signals are often more than just a byproduct of calling

Visual displays which can be presented independently of acoustic signals have been observed in several anuran species. Limb movements are used in addition to acoustic signals during courtship and male–male interaction (reviewed in H dl and Am zquita 2001; Hartmann et al. 2005). The most striking visual signalling behaviour to the human observer is foot flagging, a display during which the hind leg is raised, the toes are spread and conspicuously coloured interdigital webbings are displayed (Fig. 3). Foot flagging has been reported in 16 anuran species from five different families (H dl and Am zquita 2001; Vasudevan 2001; Hartmann et al. 2005; Krishna and Krishna 2006; Grafe and Wanger 2007). The signalling behaviour is mainly known from diurnal, stream-dwelling species (but see Am zquita and H dl 2004) and is displayed predominantly during male–male interaction or territorial encounters (H dl 1977; H dl et al. 1997; Haddad and Giarretta 1999; H dl and Am zquita 2001). In the Bornean genus *Staurois*, foot-flagging behaviour is suggested to function as an additional or alternative mode of communication in noisy stream environments. The high pitched calls of *S. guttatus*, *S. latopalmaris* and *S. parvus* are thought to alert receivers and direct their attention to the subsequent visual signal (Grafe and Wanger 2007; Preininger et al. 2009; Grafe et al. 2012). The functional separation of acoustic and visual signals allows to study influences of signal components on receivers without a determining linkage of signal modality and/or adaptation. Foot-flagging displays in the Kottigehar Dancing Frog (*M. kottigeharensis*) do not form fixed-composite signals (sensu Partan and Marler 2005) with vocalizations. Preininger et al. (2013c) suggest that the conspicuous display is a ritualization of



Fig. 3 Male *Staurois guttatus* performing the agonistic visual signal termed “foot flagging” with his left hind leg

physical attacks. Agonistic foot-flagging signals might have developed to minimize leg-kicks, a fighting technique predominantly observed in aggressive close-range encounters (Preininger et al. 2013c) and comparable to intimidation displays in bushbucks (Wronski et al. 2006; and see Smith and Evans 2011). Reactions to components of multimodal signals showed differing influences on conspecific receivers in across-species comparisons of foot-flagging species (Preininger et al. 2013b). Different signal function of multiple signal components in female mate preference was also demonstrated in squirrel tree frogs (*Hyla squirella*) and T ngara frogs (*E. pustulosus*) (Taylor et al. 2011b).

A further example of visual signalling behaviour shaped by intrasexual selection was demonstrated in recent investigations of sexual dichromatism in explosively breeding Moor frogs (*Rana arvalis*). The temporal blue nuptial colouration in males was suggested to be a visual signal promoting instantaneous mate recognition and allowing males to quickly move between rivals while scrambling for females (Ries et al. 2008; Sztatecsny et al. 2010, 2012). Nuptial dichromatism was reported for at least 31 species from seven families (Hoffmann and Blouin 2000; Bell and Zamudio 2012), however, the function of this spectacular phenomenon remains largely unexplored for most species. Hence, successful communication strategies in noisy environments can be developed in the acoustic or

visual domain, if they are beneficial for sender and receiver (reviewed in Brumm and Slabbekoorn 2005).

Visual signals in combination with or instead of acoustic signals already broaden an anuran's signal repertoire drastically, but evidence is emerging that some species utilize additional modalities.

Adding chemicals to the signal cocktail

A wide range of aquatic and terrestrial amphibians use chemical cues for orientation (Sinsch 1990; Schulte et al. 2011), prey detection (Shinn and Dole 1978; David and Jaeger 1981; Dole et al. 1981) and predator detection (Flowers and Graves 1997), which leads to the assumption that many species may have the physiological and anatomical abilities to produce and detect hetero- and conspecific chemical signals (Byrne and Keogh 2007; Woodley 2010; Hamer et al. 2011).

In aquatic and terrestrial urodeles, there are several well-known cases of chemical communication in a sexual context, such as in newts of the genus *Triturus* (Malacarne and Giacoma 1986) where chemical signals may even be more important than visual signals (Treer et al. 2013). There are few reported cases of pheromones in aquatic anurans. Females of the Magnificent tree frog (*Litoria splendida*) are attracted to the male by "splendipherin", an aquatic pheromone produced by males in glands on the head (Wabnitz et al. 1999). 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 water previously containing live males (Pearl et al. 2000). The chemicals found in *L. splendida* and in *Hymenochirus* sp. are non-volatile peptides and can, therefore, only be spread in water (Rajchard 2005; Houck 2009).

A considerable number of publications speculate about the use of skin glands present in males of many terrestrial anurans with regard to chemical communication in a sexual context due to their direct contact with the female during amplexus (Thomas et al. 1993; R del et al. 2003; Lenzi-Mattos et al. 2005; Willaert et al. 2013). However, only two cases of pheromone signals in terrestrial amphibians are presently reported. In the Australian toadlet *Pseudophryne bibronii*, 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 aid females in close-range mate localization and significantly influences male calling activity (Byrne and Keogh 2007). In mantellid frogs native to Madagascar, males have prominent femoral glands, which produce volatiles possibly acting as species-specific pheromones (Poth et al. 2012).

The use of pheromones in anuran species recognition and mate choice might be a widespread phenomenon, since

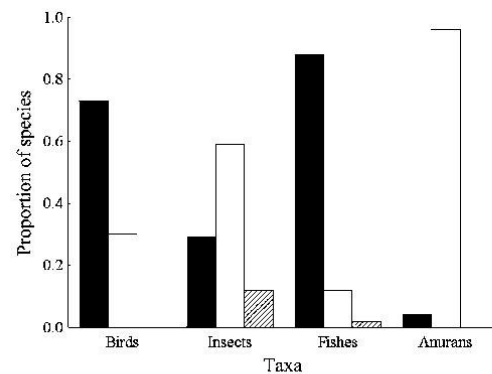


Fig. 4 Proportion of species studied within the most well-represented taxa found to use one or more of the most frequently investigated sensory modalities in mate choice (visual: solid bars; acoustic: open bars; chemical: cross-hatched bars). With kind permission from Coleman (2009), Fig. 2

chemosignals can usually be produced at low costs (Hedin et al. 1974). To date, chemical communication in anurans has been overlooked by most studies (Waldman and Bishop 2004; Belanger and Corkum 2009) in contrast to the vast number of studies on chemical communication in caudate amphibians (e.g. Vaccaro et al. 2010; Treer et al. 2013), mammals (e.g. Johnston et al. 1999) or insects (Hlldobler and Wilson 1990; Carde and Minks 1997). There is a strong disparity in the number of studies on different signal modalities in several taxa which might have led to a biased view on their signalling repertoire (Fig. 4).

The first report of possible trimodal communication in anurans comes from the species-rich frog family Hyperoliidae (Starnberger et al. 2013). Within this clade there is substantial variation in colouration, morphology, and reproductive modes, but males of most reed frog species share a common feature: a prominent gular gland on the vocal sac (Fig. 5). Chemical cocktails found in the gular gland are species specific and a combination of acoustic and chemical signals is most likely used to enhance detectability of conspecifics within dense multi-species breeding aggregations typical for hyperoliid frogs. The conspicuously coloured vocal sacs could add a further visual signalling component to the display. Hence, the vocal sac could act as a trimodal signal source which simultaneously emits acoustic, visual and chemical signals to facilitate detection, discrimination and/or location in conspecific receivers.

To understand uni- or multimodal signal efficacy we also have to question how effectively a signal influences the receiver's sensory system and subsequently its behaviour. Sophisticated experiments are needed to test, whether the

Fig. 5 (From top left to bottom right): Sympatric male individuals of *Hyperolius cin-namomeoventris*, *H. kivuensis*, *H. viridiflavus* and *H. lateralis* with inflated vocal sac. The prominent gular patch is visible in all pictures. (Photos by I. Starnberger and W. H. dl, all taken at Kibale Forest National Park, Uganda). With kind permission: Starnberger et al. (2013), Fig. 1. Copyright (2013) The Authors and The Linnean Society of London, UK



signal must be trimodal to elicit a behavioural reaction in the receiver.

The receiving end of anuran communication

Sensory perception of any signalling modality is mediated by receptors of the receiver sensory system. Mechano-, photo- and chemoreceptors react to signal stimuli and transduce the signal into electrical impulses processed by the nervous system. Acoustic vibrations of a frog's call are transmitted to two sensory organs of the inner ear specialized for the reception of sound (reviewed in Capranica 1976; Narins et al. 2007). The amphibian papilla is tonotopically organized and reacts to frequencies ranging between 80 and 1,600 Hz from the rostral to the caudal part respectively, whereas the non-tonotopic basilar papilla is sensitive to frequencies above 1,600 Hz (V lez et al. 2013). Colour and brightness, hence wavelength and intensity of perceived light, of a visual signal stimulus are processed by photoreceptors in the retina. Like most vertebrates, frogs and toads possess two types of photoreceptors—rods and cones (Kelber and Roth 2006). The anuran retina typically contains two spectral classes of rods, sensitive to the intensity of light and three spectral classes of cones, responding to colour (Bowmaker 2008). Cues or signals of airborne molecules are received by chemoreceptors in the olfactory epithelium. Anurans also possess a vomeronasal organ (Eisthen 2000) proposed to only detect non-volatile molecules (Wysocki et al. 1980; Halpern and Martinez-Marcos 2003), whereas recent studies in *Mus musculus* (Muroi et al. 2006) and *Bufo bufo* (Merkel-Harff and Ewert 1991) suggest otherwise.

In reality, several anuran species represent an exception to our extremely abridged and generalized outline of sensory transducers. A recent study for example shows that the Malagasy frog *Mantidactylus betsileanus* possesses a unique intranasal anatomy (Junk et al. 2014), which might be a development related to the frog's pheromone-producing femoral glands (Poth et al. 2012). There is also increasing evidence that many anurans have surprisingly good night vision (Cummings et al. 2008; Taylor et al. 2008; Gomez et al. 2009, 2010). *Hyla arborea* females were shown to discriminate between differently coloured male throats under nighttime light conditions, but it remains unsure, if the discrimination is based on differences in achromatic signals or if anurans are actually capable of distinguishing colours at night (but see Hailman and Jaeger 1974; Gomez et al. 2009).

Very important aspects to bear in mind when considering sexually selected traits, are not only the sensory properties of the receiver, but the actual signal components receivers respond to. In regions where *A. femoralis* calls partially overlapped with the co-occurring frog *Ameerega trivittata*, receivers of *A. femoralis* show no phonotactic reactions within the frequency range found in the call spectrum of both species (Am zquita et al. 2006). Thus, in case of sound interference by abiotic or biotic noise, it is not necessarily the signal that needs to be adapted to improve the signal-to-noise ratio. To understand the anuran sensory world in the light of sexual selection as a cause for signal adaptations, the coupled evolution of signals, sensory system, signalling behaviour and habitat choice have to be taken into account (sensory drive hypothesis) (Endler 1992; Endler and Basolo 1998). Anuran signalling traits and

behaviours are additionally affected by perceptual biases in female preference and male–male interaction particularly in regard to species recognition and predator avoidance (Ryan and Keddy-Hector 1992; reviewed in Ryan and Cummings 2013). Finally, to understand driving forces and constraints of signal design and behaviour, regardless of their uni- or multimodality, we have to investigate perceptual and cognitive mechanisms of signal processing (see Hoke et al. 2005; reviewed in Miller and Bee 2012).

Conclusion and outlook

“Until recently, efforts at understanding chorus interactions have been limited to recordings of interactions occurring over relatively small spatial scales involving just a few individuals (e.g. dyadic or triadic interactions among neighbours). Recording interactions over large spatial (and also temporal) scales was too technologically challenging, labour intensive, or both. New technological advances promise to change all this by enabling researchers to explore the complexity of chorus organization in ways only imagined in the late 1970s” (Bee et al. 2013).

As aforementioned, similar technical and conceptual advances in studies on visual and olfactory signals start to promote the research field (Cummings 2004, 2007; also see Ramsey et al. 2011). Answers to questions so elaborately proposed by Wells (1977) in the light of vocal signalling activity during social behaviour and behavioural ecology have filled textbooks (Ryan 1985; Gerhardt and Huber 2002; Narins et al. 2007) and further inspired groundbreaking research on acoustic communication systems (reviewed in Bee et al. 2013) leading from the “matched filter hypothesis” (Capranica and Moffat 1983; Gerhardt and Schwarz 2001) to the use of robotic frogs to study signal function (e.g. Narins et al. 2003; Klein et al. 2012). Today’s biological research has started to look at the big picture and integrates several sensory modalities, communication behaviour, environmental influences, perceptual biases and mechanisms of the species under investigation. Recent theoretical studies (Partan and Marler 1999, 2005; Hebets 2011) suggest different hypotheses to explain the function of signals in multimodal communication systems: multimodal or multiple signal components may evolve when they increase the signal content (content-based hypothesis), facilitate the perception of each other (inter-signal interaction hypothesis), or enhance signal transmission for instance in noisy environments (efficacy-based hypothesis) (Hebets and Papaj 2005). Based on their assumed information content, composite signals (Partan and Marler 2005) that occur together, can be further classified as redundant (all signal components elicit an equivalent response in the receiver) or non-redundant (signal components elicit a

different response in the receiver). Anuran amphibians are excellent model species to test receiver responses, e.g. via female phonotaxis or male aggressive behaviour, to stimuli in uni- and multimodal playback studies and thereby help to better understand the function of signals in relation to the proposed hypothesis. Recent investigations of anuran communication systems on isolated and combined signal components across sensory modalities also allow suggestions about perceptual processes influencing signal evolution and underlying mechanisms (Taylor and Ryan 2013).

Challenges in investigating multimodal signalling

The degree to which individual and/or combined components influence receivers in multimodal communication still remains difficult to generalize across species (Taylor et al. 2011b; Preininger et al. 2013b). To understand the efficacy of signals presented in two or more sensory modalities it is important to study the conditions in which they are presented. The efficiency of signal transmission and its effectiveness in modifying the behaviour of a receiver (Endler 2000) constitute the basis for research on signal design and evolution. Furthermore, neurobiological studies could shed much needed light on how the frog brain integrates multimodal signals (Hoke et al. 2004, 2005; Chakraborty et al. 2010; see Miller and Bee 2012; Taylor and Ryan 2013). Investigations should continue to focus on the environmental conditions which potentially favour a signal modality or their interaction and sensory modality differentiations due to detection, transmission and reception (e.g. Hoke et al. 2004). Further studies should also take into account the signal orientation of single and combined components towards same or differing receivers (e.g. mates or opponents). However, considering not only conspecific receivers but also heterospecific perceivers in the selection of signal design, we also emphasize the importance of investigations on the trade-off between efficacy and predation risk (Halfwerk et al. 2014).

We advocate that the natural history and habitat conditions of the respective study species are of utmost importance for understanding the evolution of signal design. Similarities in communication strategies do not necessarily indicate the same underlying mechanism, but could be of convergent origin and could have developed under related or different selection pressures. Interactions between sender and receiver under natural conditions could eventually help to understand the success of a certain signal and modality in the sense of signal efficacy and sensory drive.

Acknowledgments The authors express their gratitude to P. Narins, P. M. Maier and L. Landler for their helpful comments on an earlier version of this manuscript. The authors thank two anonymous reviewers for their very constructive criticism. Our study received financial support from the Austrian Science Fund (FWF): W1234 and P25612.

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standard The ethical standards of the experiments comply with the current laws of the country in which they were performed.

Open Access This article is distributed under the terms of the Creative Commons Attribution License which permits any use, distribution, and reproduction in any medium, provided the original author(s) and the source are credited.

References

- Am zquita A, H dl W (2004) How, when, and where to perform visual displays: the case of the Amazonian frog *Hyla parviceps*. *Herpetologica* 60:420–429
- Am zquita A, H dl W, Lima A et al (2006) Masking interference and the evolution of the acoustic communication system in the Amazonian dendrobatid frog *Allobates femoralis*. *Evolution* 60:1874–1887
- Bee MA (2003) A test of the “dear enemy effect” in the strawberry dart-poison frog (*Dendrobates pumilio*). *Behav Ecol Sociobiol* 54:601–610
- Bee MA (2008) Finding a mate at a cocktail party: spatial release from masking improves acoustic mate recognition in grey treefrogs. *Anim Behav* 75:1781–1791. doi:10.1016/j.anbehav.2007.10.032
- Bee MA, Gerhardt HC (2001a) Habituation as a mechanism of reduced aggression between neighboring territorial male bullfrogs (*Rana catesbeiana*). *J Comp Psychol* 115:68–82
- Bee MA, Gerhardt HC (2001b) Neighbour-stranger discrimination by territorial male bullfrogs (*Rana catesbeiana*). *Anim Behav* 62:1141–1150
- Bee MA, Schwartz JJ, Summers K (2013) All’s well that begins wells: celebrating 60 years of animal behaviour and 36 years of research on anuran social behaviour. *Anim Behav* 85:5–18
- Belanger R, Corkum L (2009) Review of aquatic sex pheromones and chemical communication in anurans. *J Herpetol* 43:184–191
- Bell RC, Zamudio KR (2012) Sexual dichromatism in frogs: natural selection, sexual selection and unexpected diversity. *Proc R Soc B* 279:4687–4693. doi:10.1242/jeb.01047
- Blair WF (1958) Mating call in the speciation of anuran amphibians. *Am Nat* 92:27–51
- Boeckle M, Preininger D, H dl W (2009) Communication in noisy environments I: acoustic signals of *Staurois latopalatus* Boulenger 1887. *Herpetologica* 65:154–165
- Bowmaker JK (2008) Evolution of vertebrate visual pigments. *Vis Res* 48:2022–2041. doi:10.1016/j.visres.2008.03.025
- Bradbury JW, Vehrencamp SL (2011) Principles of animal communication, 2nd edn. Sinauer Associates, Sunderland
- Brenowitz EA (1989) Neighbor call amplitude influences aggressive behavior and intermale spacing in choruses of the Pacific treefrog (*Hyla regilla*). *Ethology* 83:69–79. doi:10.1111/j.1439-0310.1989.tb00520.x
- Bro-Jorgensen J, Dabelsteen T (2008) Knee-clicks and visual traits indicate fighting ability in eland antelopes: multiple messages and back-up signals. *BMC Biol* 6:47. doi:10.1186/1741-7007-6-47
- Brumm H, Slabbekoorn H (2005) Acoustic communication in noise. *Adv Stud Behav* 35:151–209
- Buchanan BW (1993) Effects of enhanced lighting on the behaviour of nocturnal frogs. *Anim Behav* 45:893–899
- Bucher TL, Ryan MJ, Bartholomew GA (1982) Oxygen-consumption during resting, calling, and nest building in the frog *Physalaemus pustulosus*. *Physiol Zool* 55:10–22
- Byrne P, Keogh J (2007) Terrestrial toadlets use chemosignals to recognize conspecifics, locate mates and strategically adjust calling behaviour. *Anim Behav* 74:1155–1162
- Caldwell MS, Bee MA (2014) Spatial hearing in Cope’s gray treefrog I. Open and closed loop experiments on sound localization in the presence and absence of noise. *J Comp Physiol A* 200(4):265–284
- Caldwell MS, Johnston GR, McDaniel JG, Warkentin KM (2010) Vibrational signaling in the agonistic interactions of red-eyed treefrogs. *Curr Biol* 20:1012–1017
- Candolin U (2003) The use of multiple cues in mate choice. *Biol Rev Camb Philos Soc* 78:575–595. doi:10.1017/S1464793103006158
- Capranica RR (1976) Morphology and physiology of the auditory system. In: Llinas R, Precht W (eds) *Frog neurobiology*. Springer, New York, pp 551–575
- Capranica RR, Moffat AJM (1983) Neurobehavioral correlates of sound communication in anurans. In: Ewert J-P, Capranica RR, Ingle D (eds) *Vertebrate neuroethology*. New York Plenum, New York, pp 701–730
- Carde RT, Minks AK (1997) Insect pheromone research: new directions. Chapman & Hall, New York
- Cardoso AJ, Heyer WR (1995) Advertisement, aggressive, and possible seismic signals of the frog *Leptodactylus syphax* (Amphibia, Leptodactylidae). *Alytes* 13:67–76
- Chakraborty M, Mangiamale LA, Burmeister SS (2010) Neural activity patterns in response to interspecific and intraspecific variation in mating calls in the T ngara frog. *PLoS One* 5:e12898. doi:10.1371/journal.pone.0012898
- Coleman S (2009) Taxonomic and sensory biases in the mate-choice literature: there are far too few studies of chemical and multimodal communication. *Acta Ethol* 12:45–48
- Cummings ME (2004) Modelling divergence in luminance and chromatic detection performance across measured divergence in surfperch (*Embiotocidae*) habitats. *Vis Res* 44:1127–1145. doi:10.1016/j.visres.2003.12.013
- Cummings ME (2007) Sensory trade-offs predict signal divergence in surfperch. *Evolution* 61:530–545. doi:10.1111/j.1558-5646.2007.00047.x
- Cummings ME, Bernal XE, Reynaga R et al (2008) Visual sensitivity to a conspicuous male cue varies by reproductive state in *Physalaemus pustulosus* females. *J Exp Biol* 211:1203–1210
- David RS, Jaeger RG (1981) Prey location through chemical cues by a terrestrial salamander. *Copeia* 1981(2):435–440
- Davies NB, Halliday TR (1979) Competitive mate searching in male common toads, *Bufo bufo*. *Anim Behav* 27:1253–1267
- de Luna A, H dl W, Am zquita A (2010) Colour, size and movement as visual subcomponents in multimodal communication by the frog *Allobates femoralis*. *Anim Behav* 79:739–745
- Dickerson MC (1908) The frog book: North American toads and frogs, with a study of the habits and life histories of those of the northeastern states. Doubleday, Page and Co., Garden City
- Dole JW, Rose BB, Tachiki KH (1981) Western toads (*Bufo boreas*) learn odor of prey insects. *Herpetologica* 37:63–68
- Dorcas ME, Price SJ, Walls SC, Barichivich WJ (2010) Amphibian ecology and conservation: a handbook of techniques. In: Dodd KC (ed) *Auditory monitoring of anuran populations*. Oxford University Press, Oxford, pp 281–298
- Duellman WE, Trueb L (1986) *Biology of amphibians*. McGraw-Hill Publishing Company, New York
- Eisthen HL (2000) Presence of the vomeronasal system in aquatic salamanders. *Phil Trans R Soc B* 355:1209–1213
- Elias DO, Hebets EA, Hoy RR, Mason AC (2005) Seismic signals are crucial for male mating success in a visual specialist jumping spider (Araneae: Salticidae). *Anim Behav* 69:931–938

- Endler JA (1992) Signals, signal conditions, and the direction of evolution. *Am Nat Suppl Sens Drive* 139:S125–S153
- Endler JA (1993) Some general comments on the evolution and design of animal communication systems. *Phil Trans R Soc B* 340:215–225
- Endler JA (2000) Evolutionary implications of the interaction between animal signals and the environment. In: Espmark Y, Amundsen T, Rosenqvist G (eds) *Animal signals, signalling and signal design in animal communication*. Tapir Academic Press, Trondheim, pp 11–46
- Endler JA, Basolo AL (1998) Sensory ecology, receiver biases and sexual selection. *Trends Ecol Evol* 13:415–420
- Endler JA, Thery M (1996) Interacting effects of lek placement, display behavior, ambient light, and color patterns in three Neotropical forest-dwelling birds. *Am Nat* 148:421–452
- Feng AS, Schul J (2006) Sound processing in real-world environments. In: Narins PM, Feng AS, Fay RR, Popper AN (eds) *Hearing and sound communication in amphibians*. Springer, New York, pp 323–350
- Feng AS, Narins PM, Xu C et al (2006) Ultrasonic communication in frogs. *Nature* 440:333–336
- Flowers MA, Graves BM (1997) Juvenile toads avoid chemical cues from snake predators. *Anim Behav* 53:641–646. doi:10.1006/anbe.1996.0338
- Gasser H, Am zquita A, H dl W (2009) Who is calling? Intraspecific call variation in the arboreal frog *Allobates femoralis*. *Ethology* 115:596–607
- Gerhardt HC, Huber F (2002) *Acoustic communication in insects and anurans: common problems and diverse solutions*. University of Chicago Press, Chicago
- Gerhardt HC, Schwarz JJ (2001) Auditory tuning and frequency preferences in anurans. In: Ryan MJ (ed) *Anuran communication*. Smithsonian Institution Press, Washington DC, pp 73–85
- Gingras B, Boeckle M, Herbst CT, Fitch WT (2013) Call acoustics reflect body size across four clades of anurans. *J Zool* 289:143–150. doi:10.1038/sj.onc.1206346
- Gomez D, Richardson C, Lengagne T et al (2009) The role of nocturnal vision in mate choice: females prefer conspicuous males in the European tree frog (*Hyla arborea*). *Proc R Soc B* 276:2351–2358
- Gomez D, Richardson C, Lengagne T et al (2010) Support for a role of colour vision in mate choice in the nocturnal European treefrog (*Hyla arborea*). *Behaviour* 13:1753–1768
- Grafte TU (1996) The function of call alternation in the African reed frog (*Hyperolius marmoratus*): precise call timing prevents auditory masking. *Behav Ecol Sociobiol* 38:149–158
- Grafte TU, Wanger T (2007) Multimodal signaling in male and female foot-flagging frogs *Staurois guttatus* (Ranidae): an alerting function of calling. *Ethology* 113:772–781
- Grafte TU, Preininger D, Szatecsny M et al (2012) Multimodal communication in a noisy environment: a case study of the Bornean rock frog *Staurois parvus*. *PLoS One* 7:e37965. doi:10.1371/journal.pone.0037965
- Haddad C, Giaretta AA (1999) Visual and acoustic communication in the Brazilian torrent frog, *Hylodes asper* (Anura: Leptodactylidae). *Herpetologica* 55:324–333
- Hailman JP, Jaeger RG (1974) Phototactic responses to spectrally dominant stimuli and use of colour vision by adult anuran amphibians: a comparative survey. *Anim Behav* 22:757–795
- Halfwerk W, Jones PL, Taylor RC et al (2014) Risky ripples allow bats and frogs to eavesdrop on a multisensory sexual display. *Science* 343:413–416
- Halpern M, Martinez-Marcos A (2003) Structure and function of the vomeronasal system: an update. *Prog Neurobiol* 70:245–318. doi:10.1016/S0304-0082(03)00103-5
- Harner R, Lemckert FL, Banks PB (2011) Adult frogs are sensitive to the predation risks of olfactory communication. *Biol Lett* 7:361–363
- Hartmann MT, Giasson LOM, Hartmann PA, Haddad CFB (2005) Visual communication in Brazilian species of anurans from the Atlantic forest. *J Nat Hist* 39:1675–1685. doi:10.1080/00222930400008744
- Hebets EA (2011) Current status and future directions of research in complex signaling. *Curr Zool* 57:1–5
- Hebets E, Papaj D (2005) Complex signal function: developing a framework of testable hypotheses. *Behav Ecol Sociobiol* 57:197–214
- Hedin PA, Maxwell FG, Jenkins JN (1974) Insect plant attractants, feeding stimulants, repellents, deterrents and other related factors affecting insect behavior. In: Maxwell FG, Harris FA (eds) *Proceedings of Summer Institute on biological control of plants and diseases*. Mississippi University Press, Jackson, pp 494–527
- Hews DK, Martins EP (2013) Visual and chemical signals of social communication: providing the link to habitat and environment. In: Lutterschmidt WI (ed) *Reptiles in research*. Nova Science Publishers Inc, New York, pp 112–141
- Hirschmann W, H dl W (2006) Visual signaling in *Phrynobatrachus krefftii* Boulenger, 1909 (Anura: Ranidae). *Herpetologica* 62:18–27
- H dl W (1977) Call differences and calling site segregation in anuran species from Central Amazonian floating meadows. *Oecologia* 28:351–363
- H dl W, Am zquita A (2001) Visual signaling in anuran amphibians. In: Ryan MJ (ed) *Anuran communication*. Smithsonian Institution Press, Washington, pp 121–141
- H dl W, Rodrigues MT, Accacio GM et al (1997) Foot-flagging display in the Brazilian stream-breeding frog *Hylodes asper* (Leptodactylidae). *Austrian Federal Institute of Scientific Film (WF)*, Vienna
- Hoffmann E, Blouin MS (2000) A review of colour and pattern polymorphisms in anurans. *Biol J Linn Soc* 70:633–665
- Hoke KL, Burmeister SS, Fernald RD et al (2004) Functional mapping of the auditory midbrain during mate call reception. *J Neurosci* 24:11264–11272. doi:10.1523/JNEUROSCI.2079-04.2004
- Hoke KL, Ryan MJ, Wilczynski W (2005) Social cues shift functional connectivity in the hypothalamus. *Proc Nat Acad Sci* 102:10712–10717. doi:10.1073/pnas.0502361102
- Hlldobler B, Wilson EO (1990) *The ants*. Springer, Berlin
- Houck L (2009) Pheromone communication in amphibians and reptiles. *Annu Rev Physiol* 71:161–176
- Johnston RE, Mller-Schwarze D, Sorensen PW (1999) *Advances in chemical signals in vertebrates*. Kluwer Academic/Plenum Publishers, New York
- Junk A, Wenzel S, Vences M, Nowack C (2014) Deviant anatomy of the olfactory system of the Malagasy frog *Mantidactylus betsileanus* (Anura: Mantellidae). *Zool Anz*. doi:10.1016/j.jcz.2013.11.004
- Kelber A, Roth L (2006) Nocturnal colour vision: not as rare as we might think. *J Exp Biol* 209:781–788
- Klein BA, Stein J, Taylor RC (2012) Robots in the service of animal behavior. *Commun Integr Biol* 5:466–472. doi:10.4161/cib.21304
- Klump GM, Gerhardt HC (1992) Mechanisms and function of call-timing in male–male interactions in frogs. In: McGregor PK (ed) *Playback and studies of animal communication*. Plenum Press, New York, pp 153–174
- Krishna SN, Krishna SB (2006) Visual and acoustic communication in an endemic stream frog, *Micrixalus saxicolus* in the Western Ghats, India. *Amphib Reptil* 27:143–147

- Leal M, Fleishman LJ (2004) Differences in visual signal design and detectability between allopatric populations of *Anolis* lizards. *Am Nat* 163:26–39. doi:10.1086/379794
- Leger DW (1993) Contextual sources of information and responses to animal communication signals. *Psychol Bull* 113:295–304. doi:10.1037/0033-2909.113.2.295
- Lenzi-Mattos R, Antoniazzi M, Haddad C et al (2005) The inguinal macroglands of the frog *Physalaemus nattereri* (Leptodactylidae): structure, toxic secretion and relationship with deimatic behaviour. *J Zool* 266:385–394
- Lewis E, Narins PM, Cortopassi K et al (2001) Do male white-lipped frogs use seismic signals for intraspecific communication? *Integr Comp Biol* 41:1185–1193
- Malacarne G, Giacoma C (1986) Chemical signals in European newt courtship. *Ital J Zool* 53:79–83. doi:10.1080/11250008609355487
- McDiarmid RW, Adler K (1974) Notes on territorial and vocal behavior of neotropical frogs of the genus *Centrolenella*. *Herpetologica* 30:75–78
- Merkel-Harff C, Ewert J-P (1991) Learning-related modulation of toad's responses to prey by neural loops involving the forebrain. In: Arbib MA, Ewert J-P (eds) *Research notes in neural computing*. Springer, Berlin, pp 417–426
- Miller CT, Bee MA (2012) Receiver psychology turns 20: is it time for a broader approach? *Anim Behav* 83:331–343. doi:10.1016/j.anbehav.2011.11.025
- Muroi Y, Ishii T, Komori S et al (2006) Volatile female odors activate the accessory olfactory system of male mice without physical contact. *Neuroscience* 141:551–558
- Narins PM, Smith S (1986) Clinal variation in anuran advertisement calls: basis for acoustic isolation? *Behav Ecol Sociobiol* 19:135–141. doi:10.1007/BF00299948
- Narins PM, Zelick R (1988) The effects of noise on auditory processing and behavior in amphibians. In: Fritzsche B, Ryan MJ, Wilczynski W, Hetherington TE, Walkowiak W (eds) *The evolution of the amphibian auditory system*. Wiley, New York, pp 511–536
- Narins PM, H dl W, Grabul DS (2003) Bimodal signal requisite for agonistic behavior in a dart-poison frog, *Epipedobates femoralis*. *Proc Nat Acad Sci* 100:577–580
- Narins PM, Feng AS, Lin W et al (2004) Old World frog and bird vocalizations contain prominent ultrasonic harmonics. *J Acoust Soc Am* 115:910–913
- Narins PM, Grabul DS, Soma K et al (2005) Cross-modal integration in a dart-poison frog. *Proc Nat Acad Sci* 102:2425–2429
- Narins PM, Feng AS, Fay RR (2007) *Hearing and sound communication in amphibians*. Springer, New York
- Otovic P, Partan SR (2009) Multimodal signaling in animals. In: Squire LR (ed) *Encyclopedia of neuroscience*. Academic Press, Oxford, pp 1095–1105
- Partan S, Marler P (1999) Communication goes multimodal. *Science* 283:1272–1273
- Partan SR, Marler P (2005) Issues in the classification of multimodal communication signals. *Am Nat* 166:231–245
- Pauly GB, Bernal XE, Rand AS, Ryan MJ (2006) The vocal sac increases call rate in the T ngara frog *Physalaemus pustulosus*. *Physiol Biochem Zool* 79:708–719. doi:10.1086/504613
- Pearl C, Cervantes M, Chan M et al (2000) Evidence for a mate-attracting chemosignal in the dwarf African clawed frog *Hymenochirus*. *Horm Behav* 38:67–74
- Poth D, Wollenberg KC, Vences M, Schulz S (2012) Volatile amphibian pheromones: Macrolides from Mantellid Frogs from Madagascar. *Angew Chem Int Ed* 51:2187–2190
- Preininger D, Boeckle M, H dl W (2009) Communication in noisy environments II: visual signaling behavior of male foot-flagging frogs *Stauroids latopalmaris*. *Herpetologica* 65:166–173
- Preininger D, Boeckle M, Freudmann A et al (2013a) Multimodal signaling in the Small Torrent Frog (*Micrixalus saxicola*) in a complex acoustic environment. *Behav Ecol Sociobiol* 67:1449–1456. doi:10.1007/s00265-013-1489-6
- Preininger D, Boeckle M, Szatecsny M, H dl W (2013b) Divergent receiver responses to components of multimodal signals in two foot-flagging frog species. *PLoS One* 8:e55367. doi:10.1371/journal.pone.0055367
- Preininger D, Stiegler MJ, Gururaja KV (2013c) Getting a kick out of it: multimodal signalling during male–male encounters in the foot-flagging frog *Micrixalus aff. saxicola* from the Western Ghats of India. *Curr Sci India* 105:1735–1740
- Rajchard J (2005) Sex pheromones in amphibians: a review. *Vet Med Czech* 50:385–389
- Ramsey ME, Wong RY, Cummings ME (2011) Estradiol, reproductive cycle and preference behavior in a northern swordtail. *Gen Comp Endocr* 170:381–390. doi:10.1016/j.ygcen.2010.10.012
- Rand A, Dudley R (1993) Frogs in helium: the anuran vocal sac is not a cavity resonator. *Physiol Zool* 66:793–806
- Ries C, Spaethe J, Szatecsny M et al (2008) Turning blue and ultraviolet: sex-specific colour change during the mating season in the Balkan moor frog. *J Zool* 276:229–236
- Robertson JGM (1990) Female choice increases fertilization success in the Australian frog, *Uperoleia laevis*. *Anim Behav* 39:639–645
- R del M-O, Kosuch J, Veith M, Ernst R (2003) First record of the genus *Acanthixalus* Laurent, 1944 from the upper Guinean rain forest, West Africa, with the description of a new species. *J Herpetol* 37:43–52
- Rosenthal GG (2007) Spatiotemporal dimensions of visual signals in animal communication. *Annu Rev Ecol Syst* 38:155–178
- Rosenthal G, Rand A, Ryan MJ (2004) The vocal sac as a visual cue in anuran communication: an experimental analysis using video playback. *Anim Behav* 68:55–58
- Ryan MJ (1985) *The T ngara frog: a study in sexual selection and communication*. University of Chicago Press, Chicago
- Ryan MJ (1988) Constraints and patterns in the evolution of anuran acoustic communication. In: Fritzsche B (ed) *The evolution of the amphibian auditory system*. Wiley, New York, pp 637–677
- Ryan MJ, Cummings ME (2013) Perceptual biases and mate choice. *Annu Rev Ecol Syst* 44:437–459
- Ryan MJ, Keddy-Hector A (1992) Directional patterns of female mate choice and the role of sensory biases. *Am Nat Suppl: Sens Drive* 139:S4–S35
- Ryan MJ, Rand A (2003) Sexual selection in female perceptual space: how female T ngara frogs perceive and respond to complex population variation in acoustic mating signals. *Evolution* 57:2608–2618
- Schulte LM, Yeager J, Schulte R et al (2011) The smell of success: choice of larval rearing sites by means of chemical cues in a Peruvian poison frog. *Anim Behav* 81:1147–1154. doi:10.1016/j.anbehav.2011.02.019
- Schwartz JJ, Wells KD (1983) An experimental study of acoustic interference between two species of neotropical treefrogs. *Anim Behav* 31:181–190
- Seidel B, Yamashita M, Choi IH, Dittami J (2001) Water wave communication in the genus *Bombina* (Amphibia). *Adv Space Res* 28:589–594
- Shannon CE (1948) A mathematical theory of communication. *Bell Syst Tech J* 27:379–423
- Shinn EA, Dole JW (1978) Evidence for a role for olfactory cues in the feeding response of leopard frogs, *Rana pipiens*. *Herpetologica* 34:167–172
- Simmons AM (2012) “To Ear is Human, to Frogive is Divine”: Bob Capranica's legacy to auditory neuroethology. *J Comp Physiol A* 199:169–182. doi:10.1007/s00359-012-0786-2

- Sinsch U (1990) Migration and orientation in anuran amphibians. *Ethol Ecol Evol* 2:65–79. doi:[10.1080/08927014.1990.9525494](https://doi.org/10.1080/08927014.1990.9525494)
- Smith CL, Evans CS (2011) Exaggeration of display characteristics enhances detection of visual signals. *Behaviour* 148:287–305. doi:[10.1163/000579511X556592](https://doi.org/10.1163/000579511X556592)
- Starnberger I, Poth D, Peram PS et al (2013) Take time to smell the frogs: vocal sac glands of reed frogs (Anura: Hyperoliidae) contain species-specific chemical cocktails. *Biol J Linn Soc* 110:828–838. doi:[10.1111/bij.12167](https://doi.org/10.1111/bij.12167)
- Sztatecsny M, Strondl C, Baierl A et al (2010) Chin up: are the bright throats of male common frogs a condition-independent visual cue? *Anim Behav* 79:779–786
- Sztatecsny M, Preininger D, Freudmann A et al (2012) Don't get the blues: conspicuous nuptial colouration of male moor frogs (*Rana arvalis*) supports visual mate recognition during scramble competition in large breeding aggregations. *Behav Ecol Sociobiol* 66:1587–1593. doi:[10.1007/s00265-012-1412-6](https://doi.org/10.1007/s00265-012-1412-6)
- Taylor RC, Ryan MJ (2013) Interactions of multisensory components perceptually rescue Tungara frog mating signals. *Science*. doi:[10.1126/science.1237113](https://doi.org/10.1126/science.1237113)
- Taylor R, Buchanan B, Doherty J (2007) Sexual selection in the squirrel treefrog *Hyla squirella*: the role of multimodal cue assessment in female choice. *Anim Behav* 74:1753–1763
- Taylor R, Klein BA, Stein J, Ryan MJ (2008) Faux frogs: multimodal signalling and the value of robotics in animal behaviour. *Anim Behav* 76:1089–1097
- Taylor R, Klein BA, Stein J, Ryan MJ (2011a) Multimodal signal variation in space and time: how important is matching a signal with its signaler? *J Exp Biol* 214:815–820
- Taylor RC, Klein BA, Michael J (2011b) Inter-signal interaction and uncertain information in anuran multimodal signals. *Curr Zool* 57:153–161
- Thomas EO, Tsang L, Licht P (1993) Comparative histochemistry of the sexually dimorphic skin glands of anuran amphibians. *Copeia* 1993:133–143
- Treer D, Van Bocklaer I, Matthijs S et al (2013) Love is blind: indiscriminate female mating responses to male courtship pheromones in newts (Salamandridae). *PLoS One*. doi:[10.1371/journal.pone.0056538](https://doi.org/10.1371/journal.pone.0056538)
- Uetz G, Roberts J, Taylor P (2009) Multimodal communication and mate choice in wolf spiders: female response to multimodal versus unimodal signals. *Anim Behav* 78:299–305
- Vaccaro E, Feldhoff P, Feldhoff R, Houck L (2010) A pheromone mechanism for swaying female mate choice: enhanced affinity for a sexual stimulus in a woodland salamander. *Anim Behav* 80:983–989
- Van Staaden MJ, Smith AR (2011) Cutting the Gordian knot: complex signaling in African cichlids is more than multimodal. *Curr Zool* 57:237–252
- Vasudevan K (2001) A foot flagging frog from the Western Ghats. *Cobra* 44:25–29
- V lez A, H bel G, Gordon NM, Bee MA (2012) Dip listening or modulation masking? Call recognition by green treefrogs (*Hyla cinerea*) in temporally fluctuating noise. *J Comp Physiol A* 198:891–904. doi:[10.1007/s00359-012-0760-z](https://doi.org/10.1007/s00359-012-0760-z)
- V lez A, Schwartz JJ, Bee MA (2013) Anuran acoustic signal perception in noisy environments. In: Brumm H (ed) *Animal communication and noise*. Springer, New York, pp 133–185
- Wabnitz P, Bowie JH, Tyler M et al (1999) Animal behaviour: aquatic sex pheromone from a male tree frog. *Nature* 401:444–445
- Waldman B, Bishop PJ (2004) Chemical communication in an archaic anuran amphibian. *Behav Ecol* 15:88–93
- Wells KD (1977) The social behaviour of anuran amphibians. *Anim Behav* 25:666–693
- Wiley RH (1973) The strut display of male sage grouse: a “fixed” action pattern. *Behaviour* 47:129–152
- Willaert B, Bossuyt F, Janssenswillen S et al (2013) Frog nuptial pads secrete mating season-specific proteins related to salamander pheromones. *J Exp Biol* 216:4139–4143. doi:[10.1242/jeb.086363](https://doi.org/10.1242/jeb.086363)
- Woodley S (2010) Pheromonal communication in amphibians. *J Comp Physiol A* 196:713–727
- Wronski T, Apio A, Wanker R, Plath M (2006) Behavioural repertoire of the bushbuck (*Tragelaphus scriptus*): agonistic interactions, mating behaviour and parent–offspring relations. *J Ethol* 24:247–260
- Wysocki C, Wellington J, Beauchamp G (1980) Access of urinary nonvolatiles to the mammalian vomeronasal organ. *Science* 207:781–783. doi:[10.1126/science.7352288](https://doi.org/10.1126/science.7352288)
- Zelick R, Narins PM (1985) Characterization of the advertisement call oscillator in the frog *Eleutherodactylus coqui*. *J Comp Physiol A* 156:223–229. doi:[10.1007/BF00610865](https://doi.org/10.1007/BF00610865)

Chapter 3

INVITED REVIEW: THE ANURAN VOCAL SAC: A TOOL FOR MULTIMODAL SIGNALLING

IRIS STARNBERGER, DORIS PREININGER AND WALTER HÖDL



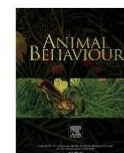
Animal Behaviour 97: 281-288

Received 24 March 2014; Accepted 16 May 2014; Published 11 September 2014



Contents lists available at ScienceDirect

Animal Behaviour

journal homepage: www.elsevier.com/locate/anbehav

Special Issue: Biochemistry & Animal Communication

The anuran vocal sac: a tool for multimodal signalling

Iris Starnberger^{a,*}, Doris Preininger^{a, b}, Walter Hödl^a^a University of Vienna, Department of Integrative Zoology, Wien, Austria^b Vienna Zoo, Wien, Austria

ARTICLE INFO

Article history:

Received 24 March 2014

Initial acceptance 16 May 2014

Final acceptance 25 July 2014

Available online 11 September 2014

MS. number: 14-00233R

Keywords:

chemical signal
functional morphology
multimodal signals
visual signal
vocal sac diversity

Although in anurans the predominant mode of intra- and intersexual communication is vocalization, modalities used in addition to or instead of acoustic signals range from seismic and visual to chemical. In some cases, signals of more than one modality are produced through or by the anuran vocal sac. However, its role beyond acoustics has been neglected for some time and nonacoustic cues such as vocal sac movement have traditionally been seen as an epiphenomenon of sound production. The diversity in vocal sac coloration and shape found in different species is striking and recently its visual properties have been given a more important role in signalling. Chemosignals seem to be the dominant communication mode in newts, salamanders and caecilians and certainly play a role in the aquatic life phase of anurans, but airborne chemical signalling has received less attention. There is, however, increasing evidence that at least some terrestrial anuran species integrate acoustic, visual and chemical cues in species recognition and mate choice and a few secondarily mute anuran species seem to fully rely on volatile chemical cues produced in glands on the vocal sac. Within vertebrates, frogs in particular are suitable organisms for investigating multimodal communication by means of experiments, since they are tolerant of disturbance by observers and can be easily manipulated under natural conditions. Thus, the anuran vocal sac might be of great interest not only to herpetologists, but also to behavioural biologists studying communication systems.

© 2014 The Authors. Published on behalf of The Association for the Study of Animal Behaviour by Elsevier Ltd. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/3.0/>).

Enormous signal diversity can be observed in animal communication systems. Physiological mechanisms are adapted over evolutionary time to generate and receive signals in various modalities (Bradbury & Vehrencamp, 2011). The courtship signals of acoustically communicating insects are produced by various stridulation processes (Gerhardt & Huber, 2002). In crickets, for example, the acoustic signals are generated by stridulation of the forewings, and the harp, a triangular anterior wing structure, acts as a sound oscillator and radiates an amplified narrow frequency band of the produced sound (Prestwich, Lenihan, & Martin, 2000). *Anolis* lizards rely almost entirely on visual signals; their colourful throat display advertises position, repels males and attracts female mates and habitat light conditions are suggested to promote signal diversity (Leal & Fleishman, 2004). Chemical signals of salamanders, pheromones produced by 'mental' chin glands, considerably increase a female's willingness to mate (Houck & Reagan, 1990; see also Vaccaro, Feldhoff, & Houck, 2010). Similar signals can be found in anuran amphibians, although the predominant mode of intra- and intersexual communication is vocalization (Dorcas, Price,

Walls, & Barichivich, 2010; Gerhardt & Huber, 2002; Ryan, 1985). It is reasonable to infer that advertisement calls of frogs, which attract females and signal readiness to defend territories to male opponents, were shaped over time through natural and sexual selection as by-products of breathing. However, during the last few decades, our view of anuran communication has broadened considerably. Anurans exhibit a striking diversity of communication strategies in the acoustic, visual, seismic and chemical domains, many of which are directly related to the so-called vocal sac, a morphological feature of most male frogs and toads (Liu, 1935).

This review aims to highlight the diversity of vocal sac morphology and function and its potential role as a 'multimodal signalling tool' in anuran communication. We further emphasize chemosignals as an additional and yet rarely investigated sensory modality in terrestrial anurans.

THE TRADITIONAL VIEW OF THE VOCAL SAC

Prior to producing a call, frogs and toads fill their lungs with air. With the mouth closed and nostrils open, they inhale by lowering the floor of the mouth, creating a negative pressure, and air flows into the oral cavity. The nostrils subsequently close and the floor of the mouth is lifted to push the air into the lungs. The ventilation

* Correspondence: I. Starnberger, University of Vienna, Department of Integrative Zoology, Althanstrasse 14, 1090 Wien, Austria.

E-mail address: iris.starnberger@univie.ac.at (I. Starnberger).

<http://dx.doi.org/10.1016/j.anbehav.2014.07.027>

0003-3472/© 2014 The Authors. Published on behalf of The Association for the Study of Animal Behaviour by Elsevier Ltd. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/3.0/>).

cycle is repeated until the lungs are sufficiently filled with air to facilitate call production. As amphibians lack ribs as well as a diaphragm and therefore are unable to passively inflate their lungs, the necessity of this procedure is apparent (Gans, 1970). Finally, to produce a call, an individual contracts its trunk muscles and air from the lungs flows via the vocal chords into the oral cavity. The airflow makes the vocal chords vibrate and a sound is produced. If frogs exhaled with each call, the lungs would rapidly be emptied and the calling individual would have to pause and again initiate the ventilation cycle. However, frogs may call for several minutes or even hours without pausing. Thus, for example, during the breeding season the New River tree frog, *Trachycephalus hadrocephalus*, is capable of producing up to 38 000 calls per night (Gaucher, 2002). This tremendous calling performance can only be achieved through an elastic skin pouch connected to the floor of the mouth which can store the air and use mechanical energy to push the air back into the lungs: the so-called vocal sac (Gans, 1973; Martin & Gans, 1972).

The anuran vocal sac probably evolved in response to selection for increasing calling efficiency (Bucher, Ryan, & Bartholomew, 1982; Pauly, Bernal, Rand, & Ryan, 2006). However, enabling a male to recycle air during calling is not the only way the vocal sac improves calling ability. Apart from fast lung reinflation it 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 et al., 1982; Pauly et al., 2006; Rand & Dudley, 1993). Calling with the mouth closed rather than open reduces the frequency range, hence narrows the bandwidth of a call and increases the intensity at the dominant frequency (Gridi-Papp, 2008). Thereby the vocal sac facilitates energetic effectiveness and acoustic conspicuousness, and is thereby also a species-specific advertisement signal.

FIRST HINTS OF A MORE COMPLEX FUNCTION

In frogs and toads, male advertisement calls play an important role in species recognition, mate choice, male spacing and territory defence (and see Toledo et al., 2014; reviewed in Wells, 1977, 1988). Thus, vocal sac movement has traditionally been seen as an epiphenomenon of call production (Dudley & Rand, 1991; Rand, 1988). Although the pulsation of the vocal sac can be interpreted as a necessary by-product of vocalization, there is increasing evidence that the visual properties of the vocal sac influence receivers and, combined with acoustic signals, form a fixed composite signal (sensu Partan & Marler, 2005). The vocal sac was first incorporated into the multimodal signal as a cue; however, colour variations and patterns could provide reliable indicators about the attributes of a sender (Gomez et al., 2009; Richardson et al., 2010; e.g. Vásquez & Pfennig, 2007) and might lead to an enhanced signal to noise ratio (Gerhardt & Schwartz, 2001).

Conspicuous white speckles on the otherwise black vocal sac of *Dendrobates pictus* clearly increase the visibility of a calling male to the human observer in a highly structured environment (Hödl, 1991). The first evidence that the pulsating vocal sac of a calling male frog might be used as a visual cue by conspecifics comes from the diurnal dart-poison frog, *Allobates femoralis* (Narins, Hödl, & Grabul, 2003). Male *A. femoralis* are highly territorial and show a stereotypic phonotaxis towards rival males calling within their territory. The phonotactic approach behaviour can be elicited by just the advertisement call and territory holders reliably locate the sound source in search of the intruder. When presented with species-specific advertisement call playbacks and a lifelike male model frog, conspecific males approached the speaker and showed no aggression towards the artificial intruder. Territory holders similarly showed no aggressive response when presented with

exclusive vocal sac pulsations; however, temporally overlapping dynamic bimodal cues provoked fighting behaviour (Narins et al., 2003; Narins, Grabul, Soma, Gaucher, & Hödl, 2005). Hence, conspecific vocalizations in *A. femoralis* elicit a phonotactic response and antiphonal calling but are not sufficient to provoke physical aggression. de Luna, Hödl, and Amézquita (2010) showed in a follow-up study that movement not only of the inflated vocal sac but also of a robotic frog model provoked territorial aggression in males of *A. femoralis*.

Similarly, the Kottigehar dancing frog, *Micrixalus kottigeharensis* (previously *Micrixalus saxicola*), a so-called foot-flagging species that performs leg waves during agonistic male interactions, only displays this behaviour in response to conspecific calls accompanied by vocal sac inflation. The visual cue was suggested to improve detection and discrimination of acoustic signals by making them more salient to receivers amid complex biotic background noise (Preininger et al., 2013).

In the East African stream frog, *Phrynobatrachus krefftii*, the conspicuous yellow vocal sac functions as a dynamic visual signal in male–male agonistic interactions even without calls being produced. The nonaudible vocal sac inflation used during male–male agonistic interactions might be a ritualized visual signal comparable to the colourful dewlaps of male *Anolis* lizards (Fitch & Hillis, 1984; Leal & Fleishman, 2004) or the striking red throat pouch in male great frigatebirds, *Fregata minor* (e.g. Juola, McGraw, & Dearborn, 2008).

Even in nocturnal species the vocal sac can be an important visual cue. Túngara frogs, *Engystomops pustulosus*, are visually sensitive at night (Cummings, Bernal, Reynaga, Rand, & Ryan, 2008) and females show a preference for advertisement calls synchronized with vocal sac inflation in video playbacks (Rosenthal, Rand, & Ryan, 2004) and robotic frog experiments (Taylor, Klein, Stein, & Ryan, 2008). Although the advertisement calls are sufficient for mate attraction, females assess multimodal stimuli during courtship. Likewise, females of the nocturnal European tree frog, *Hyla arborea*, prefer conspicuous colourful vocal sacs in addition to calls, suggesting that carotenoid-based vocal sac coloration might be a condition-dependent cue in this species (Gomez et al., 2009; Richardson, Popovici, Bellvert, & Lengagne, 2009). The availability of the vocal sac as a visual cue makes even an unattractive call with a slow call rate more appealing to females of *Hyla squirella*; however, the same result does not hold for the túngara frog, and hence added visual cues show differential modulation in female choice (Taylor, Klein, & Michael, 2011). In males of the explosively breeding common frog, *Rana temporaria*, the reflectance of the throat increases during the breeding season. The luminance of male throats, however, does not correlate with size, body condition or quality and has been suggested to be a nuptial visual cue for sex recognition in dense breeding aggregations (Sztatecsny, Ströndl, Baierl, Ries, & Hödl, 2010).

Disentangling the influence that visual cues of the multimodal signal may have on receivers remains difficult. However, detailed investigations of isolated and combined signal properties show communalities and differences between species and taxa and lead to a better understanding of not only anuran communication, but generally also of signal perception in the animal kingdom.

Moreover, the movement of the vocal sac during calling can act as a vibrational or seismic cue. In the mostly ground-dwelling white-lipped frog, *Leptodactylus albilabris* (Lewis et al., 2001) vocal sac inflation against the ground produces substrate-borne vibrations and therefore additional seismic signals to overcome the acoustic noise of a heterospecific chorus (and see Caldwell, Johnston, McDaniel, & Warkentin, 2010; Cardoso & Heyer, 1995).

The examples given above highlight the diversity in vocal sac utilization as a visual cue or signal and yet suggest that several

questions in regard to communication strategies remain to be answered.

VOCAL SAC DIVERSITY SUGGESTS UNEXPLORED FUNCTIONS

A striking diversity of vocal sac shape, size and colour can be found in frogs and toads (Fig. 1). A single subgular vocal sac seems to be the most commonly found vocal sac type (Wells, 2007); however, a similar shape does not imply a similar function.

Males of the European fire-bellied toad, *Bombina bombina*, have a subgular vocal sac, but produce sounds in the inspiratory phase. After every call their head hits the water surface thus generating waves which may be used in seismic communication (see Seidel, Yamashita, Choi, & Dittami, 2001). In *Bombina orientalis* calls are very soft, vocal sac inflation is not conspicuous and females use multimodal cues to approach a male in the water (Zeyl & Laberge, 2011). The density of water is similar to the density of the calling animal, which might lead to reduced vocal sacs or even to a lack of a vocal sac such as in the African clawed frog, *Xenopus laevis*, which calls underwater (Hayes & Krempels, 1986; e.g. Tobias et al., 2004). However, many species calling in water seem to have developed the opposite strategy by using two vocal sacs (e.g. *Pelophylax* spp., *Trachycephalus* spp.) which might facilitate floating in water and/or better airborne sound transmission (Wells, 2007). Such secondary sensory components can lead to risky consequences as recently shown in túngara frogs: water ripples produced by vocal sac inflation facilitate the localization of a calling male not only for conspecifics, but also for hunting bats, *Trachops cirrhosus* (Halfwerk, Jones, Taylor, Ryan, & Page, 2014).

Furthermore, there is increasing evidence that the vocal sac might also play a role in chemical signalling. In the family of African reed frogs (Hyperoliidae) there is substantial variation in body coloration, morphology and reproductive modes, but males of all reed frog species share a common feature: a prominent gular patch on the vocal sac, which is particularly conspicuous once the vocal



Figure 2. Male cinnamon-bellied reed frog, *Hyperolius cinnamomeiventris*, with inflated vocal sac. The gular gland and prominent blood vessels are clearly visible in the centre of the vocal sac.

sac is inflated (Fig. 2). Although the presence, shape and form of the gular patch are well-known diagnostic characters for these frogs, its function remained unknown until recently. Starnberger et al. (2013) demonstrated that the gular patch is a gland (Fig. 3) and produces species-specific volatile compound mixtures, which might be emitted while the male is calling. In the most species-rich hyperoliid genera (*Africalus*, *Heterixalus*, *Hyperolius* and *Phlyctimantis*) the proposed signal cocktails consist of 65 different compounds, whereas specific combinations of sesquiterpenes, alcohols and macrolides are correlated with species identity (Starnberger et al., 2013). Additionally, 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



Figure 1. Examples to illustrate the striking vocal sac diversity of amphibians (from left to right). First row: *Leptopelis uluguruensis*, *Bufo granulosus*, *Dendrobates pumilio*; second row: *Micrixalus kottigeharensis*, *Hyperolius cinnamomeiventris*, *Hyla meridionalis*; third row: *Hylodes phyllodes*, *Trachycephalus coriaceus*, *Allophryne ruthveni*.

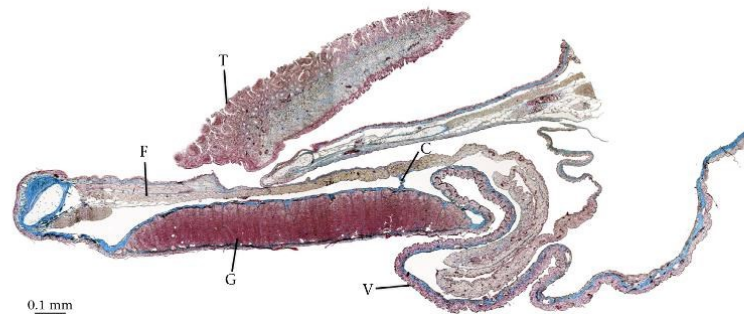


Figure 3. Histological section of the gular region of a male *Hyperolius riggenbachi* (sagittal, AZAN stain) including tongue (T) magnified by 40. The gular gland (G) tissue is clearly thicker than the surrounding vocal sac skin (V). A strand of connective tissue (C) joins the gular gland and the floor of the mouth (F).

facilitating the localization of a male calling in dense vegetation (I. Starnberger, own observations). Thus, reed frogs might use a complex combination of acoustic, visual and chemical signals in species recognition and mate choice, so far not described in any other terrestrial anuran. Hyperoliids often call in mixed choruses with closely related species without an apparent spatial segregation (Lötters et al., 2004; Schiøtz, 1999), and multimodal signals might have evolved to avoid mismating and to facilitate navigation towards a conspecific mate in dense vegetation. In one genus of the family Hyperoliidae, Rödel, Kosuch, Veith, and Ernst (2003) described two mute species which may rely solely on chemical communication via their gular glands. Several further behavioural observations suggest chemical communication in a different social context. For example, in Fausto's button frog, *Cycloramphus faustoi*, males were observed to rest their vocal sac on egg clutches, possibly to transmit pheromones that influence larval development (L.F. Toledo, personal communication). In Canebrake frogs, *Aplastodiscus perviridis*, males rest their vocal sac on females suggesting pheromone transmission during courtship (Haddad, Faivovich, & García, 2005).

Apart from the use of the anuran vocal sac, unimodal chemical signalling is widespread in amphibians but has received relatively little attention in the vast field of anuran communication (Starnberger, Preininger, & Hödl, 2014). The following section gives a brief overview of the chemical communication strategies of amphibians and of the use of chemosignals in aquatic and terrestrial environments.

CHEMICAL SIGNALLING IN AMPHIBIANS

Most amphibians have a biphasic life cycle, hence spend part of their life in water and because of their often thin and permeable skin (Duellman & Trueb, 1986) it seems reasonable that chemical signals might play an important role at least in the early stages of amphibian life histories (e.g. Jungblut, Pozzi, & Paz, 2012; Schulte et al., 2011). In aquatic and terrestrial urodeles, there are many well-known cases of chemical communication. Different skin glands in newts (Hilton, 1902; Malacarne & Giacoma, 1986; Treer et al., 2013) and salamanders (Baird, 1951; Fontana, Ask, Macdonald, Carnes, & Staub, 2006; Noble, 1929; Truffelli, 1952) release chemosignals and are used to recognize and locate partners during courtship and mating as well as opponents in territorial defence (reviewed in Woodley, 2010). Whereas chemical communication is without doubt the dominant sensory modality in urodeles, little is known about the behaviour and communication of

the vastly understudied amphibian group of caecilians (Eisthen & Polese, 2007; but see Reiss & Eisthen, 2008). A communication system based on chemosignals is suggested not only by their nocturnal and fossorial life (Waldman & Bishop, 2004), but also by the paired tentacle, an organ connected to the vomeronasal organ (Schmidt & Wake, 1990), and studies on waterborne chemical cues that attract conspecifics (Warbeck, Breiter, & Parzefall, 1996) and facilitate mate recognition (Warbeck & Parzefall, 2001).

In anuran amphibians, several tadpoles are able to detect chemical cues from predators (reviewed in Chivers & Smith, 1998; e.g. Pearl, Adams, Schuytema, & Nebeker, 2003) and chemical alarm stimuli from injured conspecifics (e.g. Hews, 1988; but also see Summey & Mathis, 1998). There are few reported cases of pheromones in adult aquatic frogs and toads. The silent and nocturnal tailed frogs, *Ascaphus truei*, live alongside noisy stream habitats, similar to foot-flagging species. Tailed frogs that were exposed to water previously containing reproductive males or females showed a preference for chemosignals of the opposite sex which suggests chemical mate recognition (Asay, Harowicz, & Su, 2005). Wabnitz, Bowie, Tyler, Wallace, and Smith (1999) found that female magnificent tree frogs, *Litoria splendida*, are attracted towards the male by 'splendipherin', an aquatic pheromone produced only by males in glands found on the head. Males of the mountain chicken frog, *Leptodactylus fallax*, secrete an aggression-stimulating peptide that provokes aggressive behaviour in males and has no effect on females (King, Rollins-Smith, Nielsen, John, & Conlon, 2005). In African clawed frogs (*Hymenochirus* sp.) females tested in Y-maze experiments showed a clear preference for water containing homogenized male postaxillary breeding glands or water previously containing live males (Pearl et al., 2000). The chemicals found in *L. splendida*, *L. fallax* and in *Hymenochirus* sp. are peptides and therefore can only be spread passively in water, but not as airborne chemical cues on land (Houck, 1998, 2009; Rajchard, 2005).

The above-mentioned studies demonstrate chemical communication as a relevant signal modality of anurans in the aquatic environment in varying social contexts.

A wide range of aquatic and also terrestrial anurans use chemical cues for navigation (Schulte et al., 2011; Sinsch, 1990) and predator detection (Flowers & Graves, 1997), which leads to the assumption that many species have the physiological and anatomical ability to produce and detect chemical signals also during terrestrial life phases (Byrne & Keogh, 2007; Woodley, 2010). During metamorphosis, the transition from water to land, anurans undergo changes in the olfactory system (Belanger & Corkum, 2009) strongly suggesting a difference in odorant access. Recent investigations

concerning adaptations of the olfactory organ in one of the most basal anuran species (*A. truei*) denote the ability to receive airborne as well as waterborne odours (Benzekri & Reiss, 2012). Belanger and Corkum (2009) described that in adult anurans the nasal cavity is capable of aquatic and terrestrial chemosignal reception. Waterborne signals are detected by the vomeronasal organ, whereas airborne odorants are processed via the principal chamber.

As for volatile signal generation, many authors have speculated on the possible use of the skin glands present in males of many terrestrial anurans. Chemical communication via skin secretions seems to be most likely in a sexual context owing to a male's direct contact with the female during mating (i.e. amplexus; Lenzi-Mattos et al., 2005; Rödel et al., 2003; e.g. Thomas, Tsang, & Licht, 1993). But to the best of our knowledge, there are only two reported cases of pheromone communication in terrestrial anurans. In the Australian toadlet, *Pseudophryne bibronii*, males call when 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 and significantly influences male calling activity (Byrne & Keogh, 2007). In Mantellid frogs native to Madagascar, males have prominent femoral glands, which produce volatiles possibly acting as species-specific pheromones (Poth, Peram, Vences, & Schulz, 2013; Poth, Wollenberg, Vences, & Schulz, 2012).

The use of pheromones in anuran species recognition and mate choice might be a widespread phenomenon, since all amphibians seem to possess a general predisposition towards the use of chemical cues (Waldman & Bishop, 2004) and since chemosignals can usually be produced at low cost (Hedin, Maxwell, & Jenkins, 1974).

Chemosignal transmission in amphibians has been suggested to occur predominantly in close-range interactions via direct contact or as short-range signals when released into water. One dispersion method is to release the odorants into a self-generated current (Bradbury & Vehrencamp, 2011) as performed by newts when flicking their tail during underwater courtship. Similar dispersion might be achieved by vocal sac pulsations which potentially fan out volatiles produced by a gular gland or even discharge a puff with each call. Without doubt, constraints such as wind could easily disturb the distribution of airborne chemical cues and therefore their localization by receivers. However, environmental conditions in the breeding swamps of calling reed frogs (i.e. high temperatures and humidity as well as elevated perching sites) would facilitate (at least) airborne short-range transmission. Thus, the inevitable vocal sac movement during calling could, apart from its visual signal content, serve to disperse chemical signals. Frogs and toads may possess more sophisticated chemical communication strategies than science has previously given them credit for, in addition to or instead of acoustic signals. To date, chemical communication in anurans has been overlooked by most studies (Belanger & Corkum, 2009; Houck, 1998, 2009; Waldman & Bishop, 2004; Woodley, 2010), probably because of the presence of more conspicuous signals (e.g. acoustic and visual) or overly costly and elaborate analyses necessary to investigate pheromones.

GUIDELINES FOR FUTURE RESEARCH

Females as well as males of many anuran species are confronted with the challenge of detecting, locating and discriminating conspecific males in breeding aggregations (Bee & Michéyl, 2008; Richardson & Lengagne, 2010) or environments that hamper propagation and transmission of acoustic signals (Boeckle, Preininger, & Hödl, 2009; Kime, Turner, & Ryan, 2000; Wells & Schwartz, 1982). Generally, signals should evolve to maximize the signal to noise ratio and to improve efficacy. Any signal detectability

depends on signal design, environmental conditions or the capacity of the medium used to transmit a signal and of course on the receiver's sensory system (Endler, 1992, 1993; Shannon, 1948). The use of multiple signal modalities, where each modality increases efficacy under specific conditions, can enhance signal conspicuousness in a variety of sensory modes (Ryan & Keddy-Hector, 1992). Misinterpreting or missing a signal is probably related to high costs. Thus a selective pressure could lead to the use of additional or alternative sensory signal modalities (Krebs & Dawkins, 1984). Furthermore, the combination of an unattractive acoustic signal and an unattractive visual signal can result in a highly attractive multimodal signal (Taylor & Ryan, 2013).

The anuran vocal sac seems likely to play a role in multimodal signalling in many more species than previously thought. Future studies should focus on describing and empirically testing the diverse functions of the vocal sac in different anuran taxa. Furthermore, there might be patterns of signal modalities and morphological features (e.g. vocal sac size) related to certain environmental conditions or habitat features (diurnal versus nocturnal, arboreal versus terrestrial versus aquatic, cue received by males, females and/or heterospecifics). To shed light on signal function, more across-species comparisons of how single and combined signal components influence receivers are needed. Furthermore, detailed descriptions of the conditions in which signals are produced are essential to draw more general conclusions on vocal sac signal function. Finally, we should question the constraints of different modalities and therefore the costs imposed by sexual or natural selection, to explain differences and similarities of this multimodal communication tool. Moreover, the majority of descriptions of amphibian glands involved in chemical courtship and mating signals lack information on the structure of the glands. This information would allow for more specific hypotheses on the origin and function of the glands described.

What Amphibians Have to Offer Research on Chemical Communication

Within vertebrates, amphibians possess a rather basal nervous system leading to a mostly stereotypic behaviour (but see for example Pasukonis, Loretto, Landler, Ringler, & Hödl, 2014; Schulte et al., 2011). Amphibians in general and frogs in particular are excellent model organisms to experimentally investigate communication strategies both in the laboratory and under natural conditions, as they are tolerant to disturbance by observers and can be easily manipulated (Hirschmann & Hödl, 2006; Klein, Stein, & Taylor, 2012; Narins et al., 2003; Taylor et al., 2008).

Amphibians show a vast diversity in reproductive modes (e.g. Hödl, 1990) and owing to their adaptations to aquatic and terrestrial habitats we can expect insights into the evolution of chemical signals in water and on land (reviewed in Woodley, 2014).

To date, a number of amphibian pheromones have already been identified (Kikuyama, Yamamoto, Iwata, & Toyoda, 2002; Poth et al., 2012; Toyoda et al., 2004; Wabnitz et al., 1999), but in comparison to the vast number of amphibian species worldwide (nearly 7300), these are probably only the tip of the iceberg.

CONCLUSION

The vast diversity of anuran vocal sacs suggests a multitude of different functions in intra- and intersexual communication shaped over evolutionary time by natural and/or sexual selection. The modalities used in addition to or instead of acoustic signals range from seismic and visual to chemical. The vocal sac, a single morphological feature, has the potential to generate multimodal signals simultaneously or sequentially. Signals of the same

modality might be directed towards and perceived by one or more conspecifics and heterospecifics. Furthermore, even signals shaped under similar evolutionary constraints may influence receivers differentially. Thus, the anuran vocal sac might be of great interest not only to herpetologists, but also to behavioural biologists investigating unimodal or multimodal communication in the acoustic, visual or chemical domains. Amphibians are highly suitable organisms to study chemical unimodal and multimodal signalling in a basal system. The focus of chemical signal research in amphibians, however, used to be mainly on urodeles. In anurans the salient acoustic and visual signals might to a certain extent have deflected our attention from equally fascinating chemical signalling strategies. Possibly, terrestrial anurans might have lost once-present chemical signals owing to the evolutionary success of acoustic communication. In some species acoustics might not have been sufficient, and thus chemical signals were 'rediscovered' and incorporated into a multimodal signal or in some species even fully replaced acoustic signals.

In this review we hope to have stimulated researchers to investigate potential chemical signals in anuran communication systems and to recognize the vocal sac as a unique multimodal signalling tool.

Acknowledgments

We thank L. F. Toledo for sharing his knowledge of extraordinary vocal sacs throughout the world, N. Haar, S. Flieger and M. Walz for their help with histological sections, P. M. Maier for helpful comments on the manuscript and two anonymous referees for their valuable input. Our study received financial support from the Austrian Science Fund (FWF): W1234 and P25612.

References

- Asay, M. J., Harowicz, P. C., & Su, L. (2005). Chemically mediated mate recognition in the tailed frog (*Ascaphus truei*). In R. T. Mason, M. P. LeMaster, & D. Müller-Schwarze (Eds.), *Chemical Signals in Vertebrates 10* (pp. 24–31). New York: Springer.
- Baird, I. L. (1951). An anatomical study of certain salamanders of the genus *Pseudoeurycea*. *University of Kansas Scientific Bulletin*, 34, 221–265.
- Bee, M. A., & Micheyl, C. (2008). The cocktail party problem: what is it? How can it be solved? and why should animal behaviorists study it? *Journal of Comparative Psychology*, 122(3), 235–251.
- Belanger, R., & Corkum, L. (2009). Review of aquatic sex pheromones and chemical communication in anurans. *Journal of Herpetology*, 43(2), 184–191.
- Benzekri, N. A., & Reiss, J. O. (2012). Olfactory metamorphosis in the coastal tailed frog *Ascaphus truei* (Amphibia, Anura, Leiopelmatidae). *Journal of Morphology*, 273(1), 68–87.
- Boeckle, M., Preininger, D., & Hödl, W. (2009). Communication in noisy environments I: acoustic signals of *Staurois latopalmatus* Boulenger 1887. *Herpetologica*, 65(2), 154–165.
- Bradbury, J. W., & Vehrencamp, S. L. (2011). *Principles of animal communication* (2nd ed.). Sunderland, MA: Sinauer Associates.
- Bucher, T. L., Ryan, M. J., & Bartholomew, G. A. (1982). Oxygen consumption during resting, calling, and nest building in the frog *Physalaemus pustulosus*. *Physiological Zoology*, 55, 10–22.
- Byrne, P., & Keogh, J. (2007). Terrestrial toadlets use chemosignals to recognize conspecifics, locate mates and strategically adjust calling behaviour. *Animal Behaviour*, 74(5), 1155–1162.
- Caldwell, M. S., Johnston, G. R., McDaniel, J. G., & Warkentin, K. M. (2010). Vibrational signaling in the agonistic interactions of red-eyed treefrogs. *Current Biology*, 20(11), 1012–1017.
- Cardoso, A. J., & Heyer, W. R. (1995). Advertisement, aggressive, and possible seismic signals of the frog *Leptodactylus syphax* (Amphibia, Leptodactylidae). *Alytes*, 13(2), 67–76.
- Chivers, D. P., & Smith, R. J. F. (1998). Chemical alarm signalling in aquatic predator-prey systems: a review and prospectus. *Ecoscience*, 5(3), 338–352.
- Cummings, M. E., Bernal, X. E., Reynaga, R., Rand, A. S., & Ryan, M. J. (2008). Visual sensitivity to a conspicuous male cue varies by reproductive state in *Physalaemus pustulosus* females. *Journal of Experimental Biology*, 211(8), 1203–1210.
- Dorcas, M. E., Price, S. J., Walls, S. C., & Barichivich, W. J. (2010). Auditory monitoring of anuran populations. In K. C. Dodd (Ed.), *Amphibian ecology and conservation: A handbook of techniques* (pp. 281–298). Oxford, U.K.: Oxford University Press.
- Dudley, R., & Rand, A. S. (1991). Sound production and vocal sac inflation in the túngara frog, *Physalaemus pustulosus* (Leptodactylidae). *Copeia*, 1991(2), 460–470.
- Duellman, W. E., & Trueb, L. (1986). *Biology of amphibians*. New York: McGraw-Hill.
- Eisthen, H. L., & Polese, G. (2007). Evolution of vertebrate olfactory subsystems. In J. H. Kaas (Ed.), *Evolution of nervous systems* (Vol. 2, pp. 355–406). Oxford, U.K.: Academic Press.
- Endler, J. A. (1992). Signals, signal conditions, and the direction of evolution. *American Naturalist, Supplement: Sensory Drive*, 139, S125–S153.
- Endler, J. A. (1993). Some general comments on the evolution and design of animal communication systems. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, 340(1292), 215–225.
- Fitch, H. S., & Hillis, D. M. (1984). The Anolis Dewlap: interspecific variability and morphological associations with habitat. *Copeia*, 2, 315–323.
- Flowers, M. A., & Graves, B. M. (1997). Juvenile toads avoid chemical cues from snake predators. *Animal Behaviour*, 53(3), 641–646.
- Fontana, M. F., Ask, K. A., Macdonald, R. J., Carnes, A. M., & Staub, N. L. (2006). Loss of traditional mucous glands and presence of a novel mucus-producing granular gland in the plethodontid salamander *Ensatina eschscholtzii*. *Biological Journal of the Linnean Society*, 87(3), 469–477.
- Gans, C. (1970). Respiration in early tetrapods – the frog is a red herring. *Evolution*, 24(4), 723–734.
- Gans, C. (1973). Sound production in the Salientia. *American Zoologist*, 13, 1179–1194.
- Gaucher, P. (2002, February 12). *Premières données sur Phrynohyas hadroceps, rainette arboricole du plateau des Guyanes (Amphibia: Anura: Hylidae) (Révision taxonomique, Éco-Ethologie de la reproduction)* (Unpublished Master's thesis). Paris, France: École pratique des hautes études, Sciences de la Vie et de la Terre.
- Gerhardt, H. C., & Huber, F. (2002). *Acoustic communication in insects and anurans: Common problems and diverse solutions*. Chicago, IL: University of Chicago Press.
- Gerhardt, H. C., & Schwartz, J. J. (2001). Auditory tuning and frequency preferences in anurans. In M. J. Ryan (Ed.), *Anuran communication* (pp. 73–85). Washington DC: Smithsonian Institution Press.
- Gomez, D., Richardson, C., Lengagne, T., Plenet, S., Joly, P., Léna, J.-P., et al. (2009). The role of nocturnal vision in mate choice: females prefer conspicuous males in the European tree frog (*Hyla arborea*). *Proceedings of the Royal Society B: Biological Sciences*, 276(1666), 2351–2358.
- Gridi-Papp, M. (2008). The structure of vocal sounds produced with the mouth closed or with the mouth open in treefrogs. *Journal of the Acoustical Society of America*, 123(5), 2895–2902.
- Haddad, C. F. B., Faivovich, J., & Garcia, P. C. (2005). The specialized reproductive mode of the treefrog *Aplastodiscus perviridis* (Anura: Hylidae). *Amphibia-Reptilia*, 26(1), 87–92.
- Halfwerk, W., Jones, P. L., Taylor, R. C., Ryan, M. J., & Page, R. A. (2014). Risky ripples allow bats and frogs to eavesdrop on a multisensory sexual display. *Science*, 343, 413–416.
- Hayes, M., & Krempel, D. (1986). Vocal sac variation among frogs of the genus *Rana* from western North America. *Copeia*, 1986(4), 927–936.
- Hedin, P. A., Maxwell, F. G., & Jenkins, J. N. (1974). Insect plant attractants, feeding stimulants, repellents, deterrents and other related factors affecting insect behavior. In F. G. Maxwell, & F. A. Harris (Eds.), *Proceedings, Summer Institute on Biological Control of Plants and Diseases* (pp. 494–527). Jackson, MI: Mississippi University Press.
- Hewes, D. K. (1988). Alarm response in larval western toads, *Bufo boreas*: release of larval chemicals by a natural predator and its effect on predator capture efficiency. *Animal Behaviour*, 36(1), 125–133.
- Hilton, W. A. (1902). A structural feature connected with the mating of *Diemictilus viridescens*. *American Naturalist*, 34, 643–651.
- Hirschmann, W., & Hödl, W. (2006). Visual signaling in *Phrynobatrachus krefftii* Boulenger, 1909 (Anura: Ranidae). *Herpetologica*, 62(1), 18–27.
- Houck, L. D. (1998). Integrative studies of amphibians: from molecules to mating. *American Zoologist*, 38(1), 108–117.
- Houck, L. D. (2009). Pheromone communication in amphibians and reptiles. *Annual Review of Physiology*, 71, 161–176.
- Houck, L. D., & Reagan, N. L. (1990). Male courtship pheromones increase female receptivity in a plethodontid salamander. *Animal Behaviour*, 39(4), 729–734.
- Hödl, W. (1990). Reproductive diversity in Amazonian lowland frogs. *Fortschritte der Zoologie*, 38, 41–60.
- Hödl, W. (1991). *Calling behaviour and spectral stratification in dart-poison frogs (Dendrobatidae)*. Wien: Austrian Federal Institute of Scientific Film (ÖWF). Retrieved from: <http://www.amphibiaweb.org>.
- Jungblut, L. D., Pozzi, A. G., & Paz, D. A. (2012). A putative functional vomeronasal system in anuran tadpoles. *Journal of Anatomy*, 221(4), 364–372.
- Juola, F. A., McGraw, K. J., & Dearborn, D. C. (2008). Carotenoids and throat pouch coloration in the great frigatebird (*Fregata minor*). *Comparative Biochemistry and Physiology, Part B*, 149(2), 370–377.
- Kikuyama, S., Yamamoto, K., Iwata, T., & Toyoda, F. (2002). Peptide and protein pheromones in amphibians. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 132(1), 69–74.
- Kime, N., Turner, W., & Ryan, M. J. (2000). The transmission of advertisement calls in Central American frogs. *Behavioral Ecology*, 11(1), 71–83.
- King, J. D., Rollins-Smith, L. A., Nielsen, P. F., John, A., & Conlon, J. M. (2005). Characterization of a peptide from skin secretions of male specimens of the frog, *Leptodactylus fallax* that stimulates aggression in male frogs. *Peptides*, 26(4), 597–601.
- Klein, B. A., Stein, J., & Taylor, R. C. (2012). Robots in the service of animal behavior. *Communicative & Integrative Biology*, 5(5), 466–472.

- Krebs, J. R., & Dawkins, R. (1984). Animal signals: mind-reading and manipulation. In J. R. Krebs, & N. B. Davies (Eds.), *Behavioural ecology: An evolutionary approach* (2nd ed., (Vol. 2, pp. 380–402). Oxford, U.K.: Blackwell.
- Leal, M., & Fleishman, L. J. (2004). Differences in visual signal design and detectability between allopatric populations of *Anolis* lizards. *American Naturalist*, 163(1), 26–39.
- Lenzi-Mattos, R., Antoniazzi, M., Haddad, C. F. B., Tambourgi, D., Rodrigues, M. T., & Jared, C. (2005). The inguinal macroglands of the frog *Physalaemus nattereri* (Leptodactylidae): structure, toxic secretion and relationship with deimatic behaviour. *Journal of Zoology*, 266(4), 385–394.
- Lewis, E., Narins, P. M., Cortopassi, K., Yamada, W., Poinar, E., Moore, W., et al. (2001). Do male white-lipped frogs use seismic signals for intraspecific communication? *Integrative and Comparative Biology*, 41(5), 1185–1193.
- Liu, C. (1935). Types of vocal sac in the Salientia. *Proceedings of the Boston Society of Natural History*, 41, 19–40.
- Lötters, S., Schick, S., Scheelke, K., Teege, P., Kosuch, J., Rotich, D., et al. (2004). Bio-sketches and partitioning of sympatric reed frogs, genus *Hyperolius* (Amphibia: Hyperoliidae), in two humid tropical African forest regions. *Journal of Natural History*, 38(15), 1969–1997.
- de Luna, A., Hödl, W., & Amézquita, A. (2010). Colour, size and movement as visual subcomponents in multimodal communication by the frog *Allobates femoralis*. *Animal Behaviour*, 79(3), 739–745.
- Malacarne, G., & Giacoma, C. (1986). Chemical signals in European newt courtship. *Italian Journal of Zoology*, 53(1), 79–83.
- Martin, W. F., & Gans, C. (1972). Muscular control of the vocal tract during release signaling in the toad *Bufo valliceps*. *Journal of Morphology*, 137(1), 1–27.
- Narins, P. M., Grabul, D. S., Soma, K., Gaucher, P., & Hödl, W. (2005). Cross-modal integration in a dart-poison frog. *Proceedings of the National Academy of Sciences*, 102(7), 2425–2429.
- Narins, P. M., Hödl, W., & Grabul, D. S. (2003). Bimodal signal requisite for agonistic behavior in a dart-poison frog, *Epipedobates femoralis*. *Proceedings of the National Academy of Sciences*, 100(2), 577–580.
- Noble, G. K. (1929). The relation of courtship to the secondary sexual characters of the two-lined salamander, *Eurycea bislineata* (Green). *American Museum of Natural History*, 362, 1–5.
- Partan, S. R., & Marler, P. (2005). Issues in the classification of multimodal communication signals. *American Naturalist*, 166(2), 231–245.
- Pašukonis, A., Loretto, M.-C., Landler, L., Ringler, M., & Hödl, W. (2014). Homing trajectories and initial orientation in a Neotropical territorial frog, *Allobates femoralis* (Dendrobatiidae). *Frontiers in Zoology*, 11(1), 29.
- Pauly, C. B., Bernal, X. E., Rand, A. S., & Ryan, M. J. (2006). The vocal sac increases call rate in the túngara frog *Physalaemus pustulosus*. *Physiological and Biochemical Zoology*, 79(4), 708–719.
- Pearl, C. A., Adams, M. J., Schuytema, G. S., & Nebeker, A. V. (2003). Behavioral responses of anuran larvae to chemical cues of native and introduced predators in the Pacific Northwestern United States. *Journal of Herpetology*, 37(3), 572–576.
- Pearl, C. A., Cervantes, M., Chan, M., Ho, U., Shoji, R., & Thomas, E. O. (2000). Evidence for a mate-attracting chemosignal in the dwarf African clawed frog *Hymenochirus*. *Hormones and Behavior*, 38(1), 67–74.
- Poth, D., Peram, P. S., Vences, M., & Schulz, S. (2013). Macrolides and alcohols as scent gland constituents of the Madagascan frog *Mantidactylus femoralis* and their intraspecific diversity. *Journal of Natural Products*, 76(9), 1548–1558.
- Poth, D., Wollenberg, K. C., Vences, M., & Schulz, S. (2012). Volatile amphibian pheromones: macrolides from mantellid frogs from Madagascar. *Angewandte Chemie International Edition*, 51, 2187–2190.
- Preininger, D., Boeckle, M., Freudmann, A., Starnberger, I., Szatecsny, M., & Hödl, W. (2013). Multimodal signaling in the small torrent frog (*Micrixalus saxicola*) in a complex acoustic environment. *Behavioral Ecology and Sociobiology*, 67(9), 1449–1456.
- Prestwich, K. N., Lenihan, K. M., & Martin, D. M. (2000). The control of carrier frequency in cricket calls: a refutation of the subalar-tegmenal resonance/auditory feedback model. *Journal of Experimental Biology*, 203(3), 585–596.
- Rajchard, J. (2005). Sex pheromones in amphibians: a review. *Veterinary Medicine - Czech*, 50(9), 385–389.
- Rand, A. S. (1988). An overview of anuran acoustic communication. In B. Fritsch, M. J. Ryan, W. Wilczynski, T. E. Hetherington, & W. Walkowiak (Eds.), *The evolution of the amphibian auditory system* (pp. 415–431). New York: J. Wiley.
- Rand, A. S., & Dudley, R. (1993). Frogs in helium: the anuran vocal sac is not a cavity resonator. *Physiological Zoology*, 66(5), 793–806.
- Reiss, J. O., & Eisthen, H. L. (2008). Comparative anatomy and physiology of chemical senses in amphibians. In J. G. M. Thewissen, & S. Nummela (Eds.), *Sensory evolution on the threshold: Adaptations in secondarily aquatic vertebrates*. Berkeley, CA: University of California Press.
- Richardson, C., & Lengagne, T. (2010). Multiple signals and male spacing affect female preference at cocktail parties in treefrogs. *Proceedings of the Royal Society B: Biological Sciences*, 277, 1247–1252.
- Richardson, C., Gomez, D., Durieux, R., Thery, M., Joly, P., Léna, J.-P., et al. (2010). Hearing is not necessarily believing in nocturnal anurans. *Biology Letters*, 6(5), 633–635.
- Richardson, C., Popovici, J., Bellvert, F., & Lengagne, T. (2009). Conspicuous colouration of the vocal sac of a nocturnal chorusing treefrog: carotenoid-based? *Amphibia-Reptilia*, 30(4), 576–580.
- Rosenthal, G. C., Rand, A. S., & Ryan, M. J. (2004). The vocal sac as a visual cue in anuran communication: an experimental analysis using video playback. *Animal Behaviour*, 68(1), 55–58.
- Rödel, M.-O., Kosuch, J., Veith, M., & Ernst, R. (2003). First record of the genus *Acanthixalus* Laurent, 1944 from the upper Guinean rain forest, West Africa, with the description of a new species. *Journal of Herpetology*, 37(1), 43–52.
- Ryan, M. J. (1985). *The túngara frog: A study in sexual selection and communication*. Chicago, IL: University of Chicago Press.
- Ryan, M. J., & Keddy-Hector, A. (1992). Directional patterns of female mate choice and the role of sensory biases. *American Naturalist, Supplement: Sensory Drive*, 139, S4–S35. JSTOR.
- Schietz, A. (1999). *Treefrogs of Africa*. Frankfurt am Main, Germany: Edition Chimaira.
- Schmidt, A., & Wake, M. H. (1990). Olfactory and vomeronasal systems of caecilians (Amphibia: Gymnophiona). *Journal of Morphology*, 205(3), 255–268.
- Schulte, L. M., Yeager, J., Schulte, R., Veith, M., Werner, P., Beck, L. A., et al. (2011). The smell of success: choice of larval rearing sites by means of chemical cues in a Peruvian poison frog. *Animal Behaviour*, 81(6), 1147–1154.
- Seidel, B., Yamashita, M., Choi, I. H., & Dittami, J. (2001). Water wave communication in the genus *Bombina* (Amphibia). *Advances in Space Research*, 28(4), 589–594.
- Shannon, C. E. (1948). A mathematical theory of communication. *Bell System Technical Journal*, 27, 379–423.
- Sinsch, U. (1990). Migration and orientation in anuran amphibians. *Ethology Ecology & Evolution*, 2(1), 65–79.
- Starnberger, I., Poth, D., Peram, P. S., Schulz, S., Vences, M., Knudsen, J., et al. (2013). Take time to smell the frogs: vocal sac glands of reed frogs (Anura: Hyperoliidae) contain species-specific chemical cocktails. *Biological Journal of the Linnean Society*, 110(4), 828–838.
- Starnberger, I., Preininger, D., & Hödl, W. (2014). From uni- to multimodality: towards an integrative view on anuran communication. *Journal of Comparative Physiology A*. <http://dx.doi.org/10.1007/s00359-014-0923-1>.
- Summey, M. R., & Mathis, A. (1998). Alarm responses to chemical stimuli from damaged conspecifics by larval anurans: tests of three neotropical species. *Herpetologica*, 402–408.
- Szatecsny, M., Ströndl, C., Baierl, A., Ries, C., & Hödl, W. (2010). Chin up: are the bright throats of male common frogs a condition-independent visual cue? *Animal Behaviour*, 79(4), 779–786.
- Taylor, R. C., Klein, B. A., & Michael, J. (2011). Inter-signal interaction and uncertain information in anuran multimodal signals. *Current Zoology*, 57, 153–161.
- Taylor, R. C., Klein, B. A., Stein, J., & Ryan, M. J. (2008). Faux frogs: multimodal signalling and the value of robotics in animal behaviour. *Animal Behaviour*, 76(3), 1089–1097.
- Taylor, R. C., & Ryan, M. J. (2013). Interactions of multisensory components perceptually rescue Túngara frog mating signals. *Science*, 341, 273–274.
- Thomas, E. O., Tsang, L., & Licht, P. (1993). Comparative histochemistry of the sexually dimorphic skin glands of anuran amphibians. *Copeia*, 1993(1), 133–143.
- Tobias, M., Barnard, C., O'Hagan, R., Horng, S., Rand, M., & Kelley, D. (2004). Vocal communication between male *Xenopus laevis*. *Animal Behaviour*, 67(2), 353–365.
- Toledo, L. F., Martins, I. A., Bruschi, D. P., Passos, M. A., Alexandre, C., & Haddad, C. F. B. (2014). The anuran calling repertoire in the light of social context. *Acta Ethologica*, 1–13.
- Toyoda, F., Yamamoto, K., Iwata, T., Hasunuma, I., Cardinali, M., Mosconi, G., et al. (2004). Peptide pheromones in newts. *Peptides*, 25(9), 1531–1536.
- Treer, D., Van Bocxlaer, I., Matthijs, S., Du Four, D., Janssenswillen, S., Willaert, B., et al. (2013). Love is blind: indiscriminate female mating responses to male courtship pheromones in newts (Salamandridae). *PLoS One*, 8(2), e56538.
- Truffelli, G. T. (1952). *A macroscopic and microscopic study of the mental hedonic gland-clusters of some plethodontid salamanders* (Unpublished doctoral thesis). U.S.A.: University of Kansas.
- Vaccaro, E., Feldhoff, P., Feldhoff, R., & Houck, L. D. (2010). A pheromone mechanism for swaying female mate choice: enhanced affinity for a sexual stimulus in a woodland salamander. *Animal Behaviour*, 80, 983–989.
- Vásquez, T., & Pfennig, K. (2007). Looking on the bright side: females prefer coloration indicative of male size and condition in the sexually dichromatic spadefoot toad, *Scaphiopus couchii*. *Behavioral Ecology and Sociobiology*, 62(1), 127–135.
- Wabnitz, P., Bowie, J. H., Tyler, M. J., Wallace, J. C., & Smith, B. (1999). Animal behaviour: aquatic sex pheromone from a male tree frog. *Nature*, 401(6752), 444–445.
- Waldman, B., & Bishop, P. J. (2004). Chemical communication in an archaic anuran amphibian. *Behavioral Ecology*, 15(1), 88–93.
- Warbeck, A., Breiter, I., & Parzefall, J. (1996). Evidence for chemical communication in the aquatic caecilian *Typhlonectes natans* (Typhlonectidae, Gymnophiona). *Mémoires de biospéologie*, 23, 37–41.
- Warbeck, A., & Parzefall, J. (2001). Mate recognition via waterborne chemical cues in the viviparous Caecilian *Typhlonectes natans* (Amphibia: Gymnophiona). In A. Marchlewska-Koj, J. J. Lepri, & D. Müller-Schwarze (Eds.), *Chemical signals in vertebrates* (Vol. 9, pp. 263–268). Boston, MA: Springer.
- Wells, K. D. (1977). The social behaviour of anuran amphibians. *Animal Behaviour*, 25, 666–693.
- Wells, K. D. (1988). The effect of social interactions on anuran vocal behavior. In B. Fritsch, W. Wilczynski, M. J. Ryan, T. E. Hetherington, & W. Walkowiak (Eds.), *The evolution of the amphibian auditory system* (pp. 433–454). New York: J. Wiley.

- Wells, K. D. (2007). *The ecology and behavior of amphibians*. Chicago, IL: The University of Chicago Press.
- Wells, K. D., & Schwartz, J. J. (1982). The effect of vegetation on the propagation of calls in the neotropical frog *Centrolenella fleischmanni*. *Herpetologica*, 38(4), 449–455.
- Woodley, S. K. (2010). Pheromonal communication in amphibians. *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology*, 196, 713–727.
- Woodley, S. K. (2014). Chemical signaling in amphibians. In C. Mucignat-Caretta (Ed.), *Neurobiology of chemical communication*. Boca Raton, FL: CRC Press.
- Zeyl, J. N., & Laberge, F. (2011). Multisensory signals trigger approach behaviour in the fire-bellied toad *Bombina orientalis*: sex differences and call specificity. *Zoology*, 114(6), 369–377.

Chapter 4

MULTIMODAL SIGNAL TESTING REVEALS GESTURAL TAPPING BEHAVIOR IN THE SPOTTED REED FROG

IRIS STARNBERGER, PHILIPP MARTIN MAIER, WALTER HÖDL AND DORIS PREININGER



Herpetologica

Received 7 September 2017, accepted for Publication 2 February 2018

Changes made to the originally submitted manuscript to fit the format of this thesis: Line numbers were deleted and figures were inserted above the respective figure caption.

Multimodal Signal Testing Reveals Gestural Tapping Behavior in the Spotted Reed Frog

STARNBERGER I.¹, MAIER P. M., HÖDL W. AND PREININGER D.²

University of Vienna, Department of Integrative Zoology, Althanstraße 14,
1090 Wien, Austria

¹ CORRESPONDENCE: e-mail, iris.starnberger@univie.ac.at

² Vienna Zoo, Maxingstraße 13b, 1130 Wien, Austria

RRH: STARNBERGER ET AL. – FOOT TAPPING IN *HYPEROLIUS PUNCTICULATUS*

ABSTRACT: In the majority of anuran species, acoustic signals are the dominant mode of inter- and intra-sexual communication. Male calls are always accompanied by the movement of a more or less conspicuous vocal sac - a potential visual cue. Reed frogs possess a striking vocal sac with a colorful patch of gland tissue clearly visible once the vocal sac is inflated during acoustic signaling. To investigate the visual signal function of vocal sac and gular gland, we presented male Spotted Reed Frogs (*Hyperolius puncticulatus*) with uni- and multimodal signal playbacks of conspecific rivals in their natural habitat and recorded behavioral responses. We found no difference in receiver response to unimodal advertisement call stimuli and to multimodal stimulus presentations of calls combined with visual signals of an artificial vocal sac with or without a gular patch, moving synchronously or asynchronously with the call playback. The inflations of a vocal sac with a colorful gular patch did not alter receiver response and neither increase nor decrease signal salience during male-male communication. The potential visual signal function of the conspicuous gular patch in inter-sexual communication remains to be tested. Interestingly, males frequently displayed a novel hind- and front foot-tapping behavior in response to all playbacks. Comparison of male responses to advertisement- and aggressive call playbacks showed that Spotted Reed Frogs approached the sound source less during aggressive call presentations. Tapping behavior was not influenced by either call playback. We suggest that the gestural tapping behavior might act as vibrational signal and discuss its potential signal function in male contests and courtship for females.

Key words: *Hyperolius*; Limb movement; Old world anurans; Tanzania; Visual display

AMPHIBIANS are excellent model systems to test uni- and multimodal communication (Starnberger et al. 2014a). Many species produce acoustic and visual signals during intraspecific communication (reviewed in Hödl and Amézquita 2001). The influence of single and combined signal components can be tested with relatively simple playback experiments in the natural environment of the species (Narins et al. 2003; Sztatecsny et al. 2012; Preininger et al. 2013a).

Acoustic signals, the dominant signal component in most anurans, provide information about body size and mass (Ryan 1988; Gingras et al. 2013), species identity (Blair 1958; Hödl 1977) and mediate sexual behavior and reproduction (Taylor et al. 2011; also see Gerhardt and Huber 2002; and Narins et al. 2007). Additional or alternative visual signals facilitate detection, recognition and discrimination in dense aggregations and noisy habitats in diurnal species (de Luna et al. 2010; Sztatecsny et al. 2012; Preininger et al. 2013a; Starnberger et al. 2014a, b). The anuran vocal sac inevitably moves as a consequence of sound production and inflates synchronously during calling. In several frog species visual cues of the inflating vocal sac make the acoustic signal more attractive to female receivers (Rosenthal et al. 2004; Cummings et al. 2008; Taylor et al. 2008; Gomez et al. 2011; Taylor and Ryan 2013) or elicit agonistic behavior and facilitate detection of opponent males (Hödl and Amézquita 2001; Narins et al. 2003; Sztatecsny et al. 2012; Preininger et al. 2013a; Starnberger et al. 2014a). In a dancing frog species (*Micrixalus kottigeharensis*) from the Western Ghats of India, vocal sac inflations combined with calls elicit visual signals such as foot flagging and hind limb tapping in perceiving males. The temporal relationship between the acoustic and visual component can influence signal attractiveness (Taylor et al. 2011; Zeyl and Laberge 2011). In Túngara frogs the visual signal of an inflating vocal sac no longer adds to the

attractiveness of the call if it is presented 100 ms or longer after the call (Taylor and Ryan 2013).

There is increasing evidence that the vocal sac might also play a role in chemical signaling. The family of African reed frogs (Hyperoliidae) exhibit substantial variation in body coloration, morphology and reproductive modes, but males of all reed frog species share a common feature: a prominent gular patch on the vocal sac (Pickersgill 2007). The conspicuous patch with high contrast to the surrounding vocal sac skin, is a gular gland complex which produces volatile substances and potentially emits scent signals during calling (Starnberger et al. 2013). Reed frogs may use a combination of acoustic, visual and chemical signals to enhance their ability to detect conspecifics within dense multi-species breeding aggregations typical for hyperoliid frogs (Ryan 1988; Taylor et al. 2011; also see Gerhardt and Huber 2002; Narins et al. 2007 and Starnberger et al. 2013).

The Spotted Reed Frog (*Hyperolius puncticulatus*, Pfeffer, 1893) is found throughout Eastern Africa (Schjötz 1999; Pickersgill 2007) and occurs in and around forests. During the breeding season males call at night from vegetation around forest pools or in swamps (Schjötz 1967). Their advertisement call consists of a series of short high-pitched notes (0.2 s) at a dominant frequency of 2.0 kHz (Schjötz 1999). Male *H. puncticulatus* emit a second call type, consisting of low rasping sounds with slow pulses (pers. observation IS, PMM and WH). Preliminary observations suggest this call is used as an aggressive call, with a similar function to the aggressive call described in other reed frog species (Passmore et al. 1992; Grafe 1995; Sinsch et al. 2011).

Complex environments can degrade signals and impair signal transmission and reception, especially in anurans using a narrow frequency bandwidth important for species specific recognition and discrimination in sexual communication. Especially high

frequency calls are more easily attenuated by heterogeneous environments (Ryan and Brenowitz 1985; Kime et al. 2000). Multimodal signal strategies, however, may allow signal transmission in all environments and thereby enhance the ability to get the message across even in nocturnal species. Some nocturnal species use visual cues and signals for species recognition and mate choice (Rosenthal et al. 2004; Richardson et al. 2010; Sztatecsny et al. 2010; Taylor et al. 2011), clearly prefer colorful vocal sacs (Gomez et al. 2009) and even discriminate colors at night (Yovanovich et al. 2017). Nevertheless for the vast majority of nocturnal anurans vocal sac inflations most likely serve to recycle air while calling for extensive periods and their visual function in the communication system remains poorly understood or might even be insignificant.

We tested the hypothesis that visual signals of the vocal sac increase signal salience in male-male interactions and influence opponents in close range male contests of nocturnal *H. puncticulatus*. To investigate the communicative function of acoustic signals and vocal sac displays we tested receiver responses to uni- and multimodal playbacks. To investigate the possible influence of fixed multimodal components on receivers, we predicted that asynchrony between the acoustic and visual components should degrade signal salience and reduce receiver response. In addition we added the visual stimulus of the gular gland to test its visual importance in the multimodal communication system in male-male interactions. Finally we used unimodal acoustic playbacks to test male receiver responses to advertisement- and aggressive calls.

MATERIALS AND METHODS

Study Site and Animals

We studied a population of *Hyperolius puncticulatus* in and around the 'Amani Pond', a swampy inundation area of the Zigi river in the Amani Nature Reserve

(5.098673°S, 38.629646°E, datum = WGS84) in the East Usambara Mountains (Tanzania) during the rainy season in August 2014. Males were abundant in the swamp and usually were spaced at a distance of at least 1 m. Males in the study population had a mean snout–urostyle length (SUL) of 26.1 mm and a mean mass of 1.01 g ($n = 12$). The frogs were captured with permission of the Amani Nature Reserve and released immediately after taking body measurements. All behavioral experiments were performed without physical contact to the focal animals.

Experimental Set-up

Focal males were located in situ by their species-specific vocalizations. In order to test their reaction to uni- and multimodal playbacks we set up a tripod (Cullman Nanomax 260) 0.5 m in front of the calling male (Fig. 1A). A bamboo stick was attached to the tripod (parallel to the ground) which held a speaker (Sony SRS-M 30). In front of the speaker a large *Asplenium* leaf was attached to the bamboo stick to serve as naturalistic platform or calling perch for playback stimuli. The artificial vocal sac was attached via an aquarium air tube (2 m long) to rubber hand bellows (Hama DustEx 00005635) to mimic rhythmic vocal sac inflations of rival males. In order to investigate the influence of the yellow gular patch as a visual signal on receivers in male-male interactions, we prepared one artificial vocal sac using a tip of a white latex glove with a central patch painted with yellow nail polish. For the second artificial vocal sac without gular patch we only used the tip of a white latex glove. The different vocal sacs could be easily exchanged between playback experiments. The speaker was connected to an mp3 player (Odys pax). The experimenter could in- and deflate the artificial vocal sac as well as start the acoustic playback from a distance of 3 m.

For acoustic stimulus presentations, we first recorded male calls with a direction-

al microphone (Sennheiser Me 66, AKG D 190 E), and a digital recorder (Zoom HN4; settings: 44.1 kHz, 16-bit resolution). We measured peak sound pressure levels (SPL) from a distance of 1 m with a Voltcraft sound pressure level meter (SL-100, settings: fast/max, C-weighted). The observed frogs ($n = 4$) produced 1–17 note calls (*median* = 2; *interquartile range* = 2) whereas 2-note calls were by far the most common. The acoustic signals of amphibians are typically time-invariant and stereotyped, showing little variation within or among individuals. We selected a 2-note vocalization for playbacks with average spectral and temporal properties representing the population mean. The average species-specific advertisement call selected for playback presentations consisted of 2 notes with a call length of 0.4 s (note length = 0.05 s) and a SPL of 78.5 dB at 1 m distance. The notes were noise reduced, sound in the narrow frequency bandwidth of notes was filtered, the inter-note interval was generated by adding a silent period of 0.3 s between notes with the sound analysis software Raven Pro to avoid interference from background noise. Similarly we recorded and analyzed aggressive calls of 4 frogs and selected an aggressive vocalization with mean parameters of the recorded individuals for the playback (call length = 0.2 s, *SPL* = 80 dB at 1 m).

1) Acoustic playback.—To investigate the function of the two call types in *Hyperolius puncticulatus* we played back advertisement- and aggressive calls and recorded male response frequencies. The first part of the acoustic stimuli consisted of 10 advertisement calls (0.4 s call time, 5.5 s inter-call interval, 53.5 s total call presentation, 80 dB at 1m), followed by a 150 s control phase (no sound), and was continued with further 10 advertisement calls and another 150 s control phase (Fig. 1B). The same advertisement call stimuli were also used for “audio” playback presentations in uni- and multimodal playbacks. To test responses to advertisement and aggressive calls the first

part of the stimulus presentation was followed by 10 aggressive calls (0.2 s call time, 2.3 s inter-call interval, 23 s total call presentation, 80dB at 1m) and a control phase (77 s) (Fig. 1B). The end of the playback and therefore of the trial was marked by 1 s of white noise, which was clearly audible in the frog chorus.

2) Uni- and multimodal playback.—Responses of a focal male were tested with one of the following randomly chosen uni- or multimodal stimuli (total $n = 39$): "audio" presentations ($n = 10$) consisted of unimodal advertisement stimuli without the artificial vocal sac and "w-sync" presentations ($n = 11$) included multimodal stimuli of the white artificial vocal sac in synchronous movement with the playback calls. Vocal sac inflations were not randomized, but followed the rhythm of the call playback. The stimulus "w-asynch" ($n = 8$) consisted of playback calls and rhythmic inflation of the white vocal sac, similar to the "w-sync"-stimulus, with the only difference that the vocal sac inflations were presented in asynchrony with the call in the inter-call interval of the audio playback. The stimulus "w-patch-sync" ($n = 10$) was identical to "w-sync" apart from the use of an artificial vocal sac with a yellow patch. As soon as the set up was positioned and the focal male stopped producing advertisement calls for more than 5 seconds, the stimulus presentation was started. The respective stimuli were presented twice for 53.5 s each time followed by a 150 s silent control period (Fig. 1C). The behavior of the focal male was recorded throughout the trial with a video camera (Sanyo Xacti WH1) for later analysis. It was not possible to record data blind because our study involved focal animals in the field. However, blinded methods were used for video analysis to minimize observer bias. After each trial, an ID photo of the focal frog's dorsal pattern (Fig. 2) was taken for individual identification to avoid retesting of males. Frequencies of all behavioral receiver responses during playback presentations and control phases were analyzed with the behavioral coding software Solomon Coder version beta 11.01. 22 (Péter

2011). The number of advertisement call responses were coded according to note number, e.g., one note-, two-note call. Calls with more than 6 notes were coded as multi-note calls. The term "advertisement call" pools the sum of all call responses to playback trials independent of note number.

Data Analysis

To test differences in behavioral response to play-back presentations of aggressive and advertisement calls, we compared response frequencies during 100 s of the first advertisement call playback and the consecutive control period to the responses displayed during 100 s of aggressive call playback presentations and the following control period. The response frequencies of "tap", "advertisement call", "aggressive call", "approach" and "move away" were analyzed using linear mixed models (LMMs). The statistical assumptions for LMM analysis were met. The LMMs allow for repeated measurements of the same individual to be fitted in the model as random variable. The respective response frequencies were entered as dependent variables, with playback presentation as predictor variable. The identities of individuals were entered as random variable.

To test the influence of uni- and multimodal playback presentations on receivers we pooled the response frequencies displayed during the complete playback (two multimodal stimulus presentations and two control periods, Fig. 1C) according to the behavioral categories of "tap", "move away", "approach", "advertisement call" and "aggressive call". Response frequencies were compared using Kruskal-Wallis Tests. To understand if the frequency of advertisement calls (i.e., the number of calls elicited) with a specific note number increases or decreases in respect to playbacks we compared one note-, two note-, three note-, four note-, five note- and multi note calls with 6 or more notes

between stimulus presentations using Kruskal-Wallis Tests. All analyses were done in SPSS version 19 (SPSS Inc., Chicago, IL, USA). Additionally, we analyzed recordings of advertisement calls and aggressive calls in Raven Pro 1.3 (build 32) software for Windows.

RESULTS

Call Descriptions

The advertisement call of *H. puncticulatus* in the study population consisted of one to sixteen notes with two-note calls being the most common. One note had an average duration of 0.055 s ($SE \pm 0.002$; *range*: 0.043 - 0.066; $n = 12$) and a dominant frequency of 2982 Hz ($SE \pm 33$; *range*: 2832 - 3182; $n = 12$). The aggressive call is pulsatile with an average call duration of 0.22 s ($SE \pm 0.019$; *range*: 0.11 - 0.36; $n = 12$) and a dominant frequency of 2975 Hz ($SE \pm 36$; *range*: 2815 - 3167; $n = 12$; Fig. 4). Our observations of pulsatile aggressive calls and variable multi-note advertisement calls correspond to those reported for other *Hyperolius* calls (Grafe 1995, 1996; Schiøtz 1999). The average call duration of a two note advertisement call (0.51 s; $SE \pm 0.01$; *range*: 0.46 - 0.60; $n = 12$) in the study population was twice as long as the overall call duration reported by Schiøtz (1999). If a male approaches another calling male, the calling male is likely to switch from producing advertisement calls to aggressive calls. If the intruding male does not move away, the resident male will often engage in agonistic behavior such as fighting.

Behavioral Responses to Playback Presentations

Of 3482 recorded behaviors in response to uni- and multimodal playback presentations, 49 % of the displayed behaviors of male Spotted Reed Frogs were advertise-

ment calls, 10 % aggressive calls, 6% approaches to the sound source and 3% movement away from it. Additionally, males displayed a yet undescribed behavior for this species, which we termed "tapping", in a surprisingly high frequency. Tapping denotes the brief lifting of a limb (i.e. one arm or leg) from the substrate without stretching or waving. Thirty-two percent of behavioral responses were taps of either the right or left front or hind limb. Especially hind limbs used for tapping displays were frequently switched. Tapping could only be observed in males and was mostly displayed in addition to advertisement calls (also see enclosed Video 1 of a male displaying the described behavior during an experimental trial in the supplemental files).

1) Acoustic playback.—*Hyperolius puncticulatus* approached advertisement call playbacks more readily than aggressive call playbacks ($F_{1/18} = 5.00$; $P = 0.04$; Fig. 4). While aggressive call playbacks tended to be answered less often with advertisement calls than advertisement call playbacks, this difference was not significant ($F_{1/18} = 3.47$; $P = 0.08$). We found no differences in "tap"- ($F_{1/18} = 0.53$; $P = 0.48$), "aggressive call"- ($F_{1/18} = 1.03$; $P = 0.32$) or "move away" responses ($F_{1/18} = 0.58$; $P = 0.46$) to advertisement- and aggressive call playback presentations ($F_{1/18} = 4.23$; $P = 0.06$), for each stimulus presentation $n = 10$.

2) Uni- and multimodal playback.—Behavioral reactions of male *H. puncticulatus* did not differ in response to uni- and multimodal playbacks (Fig. 5). Focal males displayed tapping behavior (Kruskal Wallis Test: $H = 0.32$; $df = 3$, $P = 0.96$), produced advertisement calls (Kruskal Wallis Test: $H = 3.15$; $df = 3$, $P = 0.37$) and aggressive calls ($H = 1.76$; $df = 3$, $P = 0.62$; Fig. 6) and approached the stimulus (Kruskal Wallis Test: $H = 0.18$; $df = 3$, $P = 0.98$) or moved away from it (Kruskal Wallis Test: $H = 1.76$; $df = 3$, P

= 0.62) in similar frequencies in response to all four playback types. We found no difference in number of one note calls, two note calls or multi note calls between stimulus presentations, hence males did not increase or decrease call duration by emitting more or less notes per advertisement call in response to a playback presentation (Kruskal Wallis Tests: $df = 3$, $P > 0.05$ for all tested notes per call).

Field Observations on Courtship Behavior in *Hyperolius puncticulatus*

We observed the interaction between a male and a gravid female in the study population. The female moved through the vegetation approaching the advertising male and then perched on a leaf in the vicinity of the calling male. Eggs were partly visible through the translucent skin on the female's ventrum. The female stayed motionless for several minutes, while the male emitted advertisement calls and frequently tapped using front and hind limbs. The female then jumped towards the calling male and turned on the spot to face away from him. The male approached the female while continuously emitting advertisement calls and rapidly jumped onto her back into amplexus. The amplexant pair then left the calling site. Video 2 in the supplemental files shows a section of the observed seven minute courting and mating behavior leading to amplexus.

DISCUSSION

In *Hyperolius puncticulatus* the vocal sac, moving inevitably during advertising, did not act as a visual display which increases agonistic response behavior (e.g., approaches, attacks or aggressive calls) towards conspecific males, regardless of the presence or absence of the gular patch. Receiver response was not reduced when calls and vocal sac inflations were presented in asynchrony, even though this fixed composite signal sensu Partan and Marler (2005) exclusively occurs in synchrony as a biome-

chanical consequence of call production. Acoustic and visual signal components, although temporally offset, could be still recognized and detected by receivers, because signal saliency relies on the relative relationship of signal components (Rubi and Stephens 2016; Stange et al. 2016). Several studies have demonstrated that multimodal signals enhance detection and recognition of the signaler (Preininger et al. 2013a; Taylor and Ryan 2013; and reviewed in Starnberger et al. 2014a) and components act as back-up or alert receivers in fluctuating noisy environments (e.g., Partan and Marler 2005). However, if receivers rely on the comparison of multimodal components in mate choice, fluctuating environmental conditions can cause signal interference (Taylor et al. 2011; Zeyl and Laberge 2011; Halfwerk and Slabbekoorn 2015; Halfwerk et al. 2016). Unlike females, male reed frogs might not depend on precisely locating another male, but rather estimate caller distance or calls might simply help to detect the presence of a nearby rival. When nearby male *H. puncticulatus* move too close to a male's calling perch, resident males switch from advertisement- to aggressive calls. In response to unimodal aggressive call playbacks, the focal male usually stopped approaching. A consistent stimulus presentation of two advertisement calls followed by one aggressive call was used during playback presentations to mimic a natural call sequence of a conspecific male in close range. We chose this call sequence to prevent influence of aggressive calls on advertisement call responses but have to consider that testing males with only aggressive calls versus only advertisement call presentations and/or a higher sample size might have provided differing responses or response frequencies. Thereby interpretations of the results infer to the successive call presentation and provide only initial information for future investigations. Nevertheless we suggest *H. puncticulatus* switch to aggressive calls to defend perching sites and conspecifics stop their approach to avoid attacks from the resident male.

Aggressive calls have generally been suggested to play a role in male-male-spacing (Taylor and Ryan 2013; Toledo et al. 2015) and in assessing a males quality, but compared to advertisement calls they are most likely unattractive to females (Wells 1988; Grafe 1995; Pickersgill 2007 and see Marshall, Humfeld and Bee 2003). Therefore, eliciting aggressive calls might come at the cost of not being able to produce advertisement calls (Passmore et al. 1992; Starnberger et al. 2013), which attract females and signal the readiness to defend a calling site (Narins et al. 2007).

Our results suggest, that visual cues of the vocal sac and gular patch can be considered insignificant in the male-male communication system of *H. puncticulatus*. Even though responses of only eight to eleven males were tested, our results demonstrate no differences in response frequencies to uni- or multimodal playback presentations. We believe, that an increased sample size would not alter the results. However the vocal sac was presented without an attached model frog. Therefore, we suggest future investigations should test visual signal response to inflations in the context of a frog model. Furthermore, it is still unclear if the movement of the vocal sac increases the detectability of a male eliciting aggressive calls, or could be used as agonistic signal at a close range. Additionally we have to consider the potential role of visual cues in female choice. On one hand, a high contrast between vocal sac and gular patch could facilitate detection of a calling male in dense vegetation (Starnberger et al. 2014b). On the other hand, the visual properties of the vocal sac might provide information about a male's health status and overall condition. In the nocturnal tree frog *Hyla arborea* females prefer more intensely colored vocal sacs, suggesting that carotenoid-based yellow to orange coloration might be a condition-dependent cue (Gomez et al. 2009, 2011). Gravid reed frog females have a high risk of predation due to the increased activity during mate search. Preference for very conspicuous and therefore easily locatable males may be beneficial

(Grafe 1996, 1997; Schiøtz 1999; Pickersgill 2007). Conspicuousness is often achieved with energetically expensive high call rates and high sound pressure levels in anurans (Schiøtz 1967; Klump and Gerhardt 1992). *Hyperolius marmoratus* females prefer nearby males eliciting advertisement calls at a high rate (Grafe 1997; Schiøtz 1999). Female reed frogs might potentially use concomitant visual vocal sac cues to assess mates similar to *Engystomops pustulosus*, where gravid females are most sensitive to the visual cue (Cummings et al. 2008). Sexual selection could also have favored a close range signal in a different signal domain with slower, but more constant transmission such as a chemical cue.

In male reed frogs, the gular patch is a colorful gland situated on the vocal sac with ducts leading to the outside. Volatile chemicals produced in the gland might be radiated during calling (Starnberger et al. 2013). Species-specific scent-bouquets might act as courtship signal comparable to pheromones distributed by newts in a self-generated current (Ryan and Brenowitz 1985; Kime et al. 2000; Woodley 2010; Bradbury and Vehrencamp 2011). During the nocturnal courtship of *H. puncticulatus* we observed a vocal sac remaining inflated for more than 6 min before amplexus was initiated (see Video 2 in the supplemental files). Evidence about the function of the gular gland remains anecdotal, but it seems likely, that the transmission and perception of volatile chemicals could be more effective than visual signals under low light conditions (Grafe 1995, 1996; Schiøtz 1999; Waldman and Bishop 2004; Byrne and Keogh 2007).

Spotted Reed Frogs displayed a yet undescribed behavior in response to playbacks, a gestural display. A series of front and hind limb lifts or tapping behaviors were displayed in response to playback presentations. Even though no differences in the frequency of tapping were recorded in regard to playback presentations, tapping and advertisement calls were the most frequently displayed behaviors of males. Visual signals

of hind foot- and arm lifting or waving were so far described as agonistic behavior in the signal repertoire of anurans (Hödl and Amézquita 2001). Even more conspicuous gestural displays like foot-flagging behavior were only recorded in an agonistic context during male-male interactions to defend perching sites (Amézquita and Hödl 2004; Preininger et al. 2009; Grafe et al. 2012) and are suggested to originate from an aggressive kicking behavior used during physical attacks (Preininger et al. 2013b). Kicking bouts are also displayed by male *Hyperolius marmoratus* in defense of conspecific intruders (Grafe 1997) and males in several reed frogs species use physical combat to defend their position in the lek (Telford 1985; Dyson et al. 1992). Male spacing increases mating success, because females prefer males with larger sound fields and cannot detect overlapping calls (Grafe 1995, 1996). Limb displays in *H. puncticulatus* could be an agonistic visual signal for perceiving opponents. We also observed tapping behavior in the presence of a female and the display was not regarded aversive, on the contrary the signaling male mated with the female (Video 2). Thereby we suggest tapping behavior might attract females and repel males similar to advertisement calls.

Tapping however might not stimulate the visual system of conspecifics, but influence a different sensory modality via substrate vibrations in a limited active signal space.

Seismic or vibrational signals could act as close-range communication channel in arboreal anurans, when the efficacy of visual signal is obscured by the environment. Similar to our study species, arboreal and nocturnal Red-eyed Treefrogs (*Agalychnis callidryas*) form breeding aggregations in vegetation's around water bodies or lakes. Males produce tremulations to create substrate vibrations used as aggressive signals carrying information about the motivation and size of the signaler (Caldwell et al. 2010). Anurans also use vibrational signals to attract mates. Females of the South Asian common Treefrog (*Polypedates leucomystax*) tap their toes to initiate mating (Narins et al.

1998). Males of the ground-dwelling White-lipped Frog (*Leptodactylus albilabris*) generate seismic signals with their vocal sac while calling (Narins 1990; Lewis et al. 2001; Gridi-Papp and Narins 2010) and male *Leptodactylus syphax* respond to aggressive calls with a combination of aggressive calls and audible foot taps (Cardoso and Heyer 1995). We suggest vibrational and/or visual tapping signals could facilitate and promote detection in complex or heterogeneous environments.

Conclusion

In anurans, research in the past decade has provided valuable information on perception of and behavioral response to multimodal signals with components either always produced together (i.e., fixed components) or independently. Furthermore, a lot of effort has been put forward to describe environmental influences and the selection on multimodal signal design in several species. However, not all species respond to or use the available modalities for communication. Admittingly, this study focused on responses to multimodal stimuli during male-male interactions, but we would still like to suggest that conspicuously moving vocal sacs could sometimes just be a byproduct of sound production and selection might not promote visual signal components in nocturnal species. Nevertheless, vocal sac pulsations could facilitate a less explored modality in anurans and might be used to radiate chemical signals from the gular gland. Future investigations should focus on chemical cues emitted by the gular patch and test receiver responses of females to visual and chemical signals. The novel gestural tapping display also raises several questions in regard to its function in male-male interaction and female mate choice. Spotted Reed Frogs provide the opportunity to test the effectiveness and complexity of signals in several sensory modalities in a nocturnal species.

Acknowledgments. —We thank P. M. Narins for helpful comments on the experimental set-up, N. Kavcik-Graumann for her illustration of the set-up, the Tropical Biology Association (especially C. Nuttman, P. Gacheru and M. Kamoga for their tremendous help with logistics and the Amani Nature Reserve for establishing the landowners' support for our field work, the Austrian Science Fund (FWF): P25612 and W1234 for financial support and K. Wadsworth, S. J. Mullin and two anonymous reviewers for their constructive comments concerning the manuscript. We confirm, that appropriate protocols were followed in the handling and observation of the subjects of this study and that all contributors observed appropriate ethical and legal guidelines and regulations.

LITERATURE CITED

- Amézquita, A., and W. Hödl. 2004. How, when, and where to perform visual displays: the case of the Amazonian frog *Hyla parviceps*. *Herpetologica* 60:420–429.
- Blair, W.F. 1958. Mating call in the speciation of anuran amphibians. *American Naturalist* 92:27–51.
- Bradbury, J.W., and S.L. Vehrencamp. 2011. *Principles of Animal Communication*. 2nd ed. Sinauer Associates, United Kingdom.
- Byrne, P.G., and J.S. Keogh. 2007. Terrestrial toadlets use chemosignals to recognize conspecifics, locate mates and strategically adjust calling behaviour. *Animal Behaviour* 74:1155–1162.
- Caldwell, M.S., G.R. Johnston, J.G. McDaniel, and K.M. Warkentin. 2010. Vibrational signaling in the agonistic interactions of red-eyed treefrogs. *Current Biology* 20:1012–1017.

- Cardoso, A.J., and W.R. Heyer. 1995. Advertisement, aggressive, and possible seismic signals of the frog *Leptodactylus syphax* (Amphibia, Leptodactylidae). *Alytes* 13:67–76.
- Cummings, M.E., X.E. Bernal, R. Reynaga, A.S. Rand, and M.J. Ryan. 2008. Visual sensitivity to a conspicuous male cue varies by reproductive state in *Physalaemus pustulosus* females. *Journal of Experimental Biology* 211:1203–1210.
- de Luna, A., W. Hödl, and A. Amézquita. 2010. Colour, size and movement as visual subcomponents in multimodal communication by the frog *Allobates femoralis*. *Animal Behaviour* 79:739–745.
- Dyson, M.L., N.I. Passmore, P.J. Bishop, and S.P. Henzi. 1992. Male behavior and correlates of mating success in a natural population of African painted reed frogs (*Hyperolius marmoratus*). *Herpetologica* 236–246.
- Gerhardt, H.C., and F. Huber. 2002. *Acoustic Communication in Insects and Anurans: common problems and diverse solutions*. University of Chicago Press, USA.
- Gingras, B., M. Boeckle, C.T. Herbst, and W.T. Fitch. 2013. Call acoustics reflect body size across four clades of anurans. *Journal of Zoology* 289:143–150. DOI: <https://doi.org/10.1038/sj.onc.1206346>.
- Gomez, D., C. Richardson, T. Lengagne, S. Plenet, P. Joly, J.-P. Léna, and M. Thery. 2009. The role of nocturnal vision in mate choice: females prefer conspicuous males in the European tree frog (*Hyla arborea*). *Proceedings of the Royal Society of London. Series B: Biological Sciences* 276:2351–2358.

- Gomez, D., M. Thery, A.-L. Gauthier, and T. Lengagne. 2011. Costly help of audiovisual bimodality for female mate choice in a nocturnal anuran (*Hyla arborea*). *Behavioral Ecology* 22:889–898. DOI: <https://doi.org/10.1093/beheco/arr039>.
- Grafe, T.U. 1995. Graded aggressive calls in the African painted reed frog *Hyperolius marmoratus* (Hyperoliidae). *Ethology* 101:67–81. DOI: <https://doi.org/10.1080/09524622.1988.9753071>.
- Grafe, T.U. 1996. The function of call alternation in the African reed frog (*Hyperolius marmoratus*): precise call timing prevents auditory masking. *Behavioral Ecology and Sociobiology* 38:149–158. DOI: <https://doi.org/10.1007/s002650050227>.
- Grafe, T.U. 1997. Costs and benefits of mate choice in the Lek-breeding reed frog, *Hyperolius marmoratus*. *Animal Behaviour* 53:1103–1117.
- Grafe, T.U., D. Preininger, M. Sztatecsny, R. Kasah, J.M. Dehling, S. Proksch, and W. Hödl. 2012. Multimodal communication in a noisy environment: a case study of the Bornean rock frog *Staurois parvus*. *PLoS ONE* 7:e37965. DOI: <https://doi.org/10.1371/journal.pone.0037965>.
- Gridi-Papp, M., and P.M. Narins. 2010. Seismic Detection and Communication in Amphibians. Pp. 69–83 in *The use of Vibrations in Communication: Properties, Mechanisms and Function across Taxa* (C. O’Connell-Rodwell, ed.). Transworld, India.
- Halfwerk, W., and H. Slabbekoorn. 2015. Pollution going multimodal: the complex impact of the human-altered sensory environment on animal perception and performance. *Biology Letters* 11:20141051–20141051. DOI: <https://doi.org/10.1098/rspb.2008.0163>.

- Halfwerk, W., M.J. Ryan, and P.S. Wilson. 2016. Wind- and rain-induced vibrations impose different selection pressures on multimodal signaling. *The American Naturalist* 188:279–288.
- Hödl, W. 1977. Call differences and calling site segregation in anuran species from Central Amazonian floating meadows. *Oecologia* 28:351–363.
- Hödl, W., and A. Amézquita. 2001. Visual Signaling in anuran Amphibians. Pp. 121–141 in *Anuran Communication* (M.J. Ryan, ed.). Smithsonian Institution Press, USA.
- Kime, N., W. Turner, and M.J. Ryan. 2000. The transmission of advertisement calls in Central American frogs. *Behavioral Ecology* 11:71–83.
- Klump, G.M., and H.C. Gerhardt. 1992. Mechanisms and Function of Call-timing in male-male Interactions in Frogs. Pp. 153–174 in *Playback and Studies of Animal Communication* (P.K. McGregor, ed.). Plenum Press, USA.
- Lewis, E., P.M. Narins, K. Cortopassi, W. Yamada, E. Poinar, W. Moore, and X. Yu. 2001. Do male white-lipped frogs use seismic signals for intraspecific communication? *Integrative and Comparative Biology* 41:1185–1193.
- Marshall, V.T., S.C. Humfeld, and M.A. Bee. 2003. Plasticity of aggressive signalling and its evolution in male spring peepers (*Pseudacris crucifer*). *Animal Behaviour* 65:1223–1234.
- Narins, P.M. 1990. Seismic communication in anuran amphibians. *Bioscience* 40:268–274.

- Narins, P.M., A.S. Feng, and H.S. Yong. 1998. Morphological, behavioral, and genetic divergence of sympatric morphotypes of the treefrog *Polypedates leucomystax* in peninsular Malaysia. *Herpetologica* 54:129–142.
- Narins, P.M., W. Hödl, and D.S. Grabul. 2003. Bimodal signal requisite for agonistic behavior in a dart-poison frog, *Epipedobates femoralis*. *Proceedings of the National Academy of Sciences* 100:577–580.
- Narins, P.M., A.S. Feng, and R.R. Fay. 2007. *Hearing and Sound Communication in Amphibians*. Springer, USA.
- Partan, S.R., and P. Marler. 2005. Issues in the classification of multimodal communication signals. *The American Naturalist* 166:231–245.
- Passmore, N.I., P.J. Bishop, and N. Caithness. 1992. Calling behaviour influences mating success in male painted reed frogs, *Hyperolius marmoratus*. *Ethology* 92:227–241.
- Péter, A. 2011. (version beta 11.01. 22): Solomon Coder: a simple solution for behavior coding. Computer programm available at <http://solomoncoder.com>.
- Pickersgill, M. 2007. *Frog Search: Results of Expeditions to Southern and Eastern Africa from 1993-1999*. Edition Chimaira, Germany.
- Preininger, D., M. Boeckle, and W. Hödl. 2009. Communication in noisy environments II: Visual signaling behavior of male foot-flagging frogs *Staurois latopalmatus*. *Herpetologica* 65:166–173.

- Preininger, D., M. Boeckle, A. Freudmann, I. Starnberger, M. Sztatecsny, and W. Hödl. 2013a. Multimodal signaling in the small torrent frog (*Micrixalus saxicola*) in a complex acoustic environment. *Behavioral Ecology and Sociobiology* 67:1449–1456. DOI: <https://doi.org/10.1007/s00265-013-1489-6>.
- Preininger, D., M.J. Stiegler, and K.V. Gururaja. 2013b. Getting a kick out of it: multimodal signalling during male–male encounters in the foot-flagging frog *Micrixalus* aff. *saxicola* from the Western Ghats of India. *Current Science* 105:1735–1740.
- Richardson, C., D. Gomez, R. Durieux, M. Thery, P. Joly, J.-P. Léna, S. Plenet, and T. Lengagne. 2010. Hearing is not necessarily believing in nocturnal anurans. *Biology Letters* 6:633–635.
- Rosenthal, G.G., A.S. Rand, and M.J. Ryan. 2004. The vocal sac as a visual cue in anuran communication: an experimental analysis using video playback. *Animal Behaviour* 68:55–58.
- Rubi, T.L., and D.W. Stephens. 2016. Why complex Signals matter, sometimes. Pp. 119–135 in *Psychological Mechanisms in Animal Communication* (M.A. Bee and C.T. Miller, eds.). Springer International Publishing, Switzerland.
- Ryan, M.J. 1988. Constraints and Patterns in the Evolution of anuran acoustic communication. Pp. 637–677 in *The Evolution of the Amphibian Auditory System* (B. Fritzsch, ed.). John Wiley and Sons, USA.
- Ryan, M.J., and E.A. Brenowitz. 1985. The Role of Body Size, Phylogeny, and Ambient Noise in the Evolution of Bird Song. *American Naturalist* 126:87–100.

- Schiøtz, A. 1967. The treefrogs (Rhacophoridae) of West Africa. *Spolia Zoologica Musei Hauniensis* 25:1–346.
- Schiøtz, A. 1999. Treefrogs of Africa. Edition Chimaira, Germany.
- Sinsch, U., E. Greenbaum, C. Kusamba, and E. Lehr. 2011. Rapid assessment of montane anuran communities in the Albertine Rift: *Hyperolius castaneus* Ahl, 1931 as an umbrella species for conservation. *African Zoology* 46:320–333.
- Stange, N., R.A. Page, M.J. Ryan, and R.C. Taylor. 2016. Interactions between complex multisensory signal components result in unexpected mate choice responses. *Animal Behaviour* DOI: <https://doi.org/10.1016/j.anbehav.2016.07.005>.
- Starnberger, I., D. Poth, P.S. Peram, S. Schulz, M. Vences, J. Knudsen, M.F. Barej, M.-O. Rödel, M. Walzl and W. Hödl. 2013. Take time to smell the frogs: vocal sac glands of reed frogs (Anura: Hyperoliidae) contain species-specific chemical cocktails. *Biological Journal of the Linnean Society* 110:828–838. DOI: <https://doi.org/10.1111/bij.12167>.
- Starnberger, I., D. Preininger, and W. Hödl. 2014a. From uni- to multimodality: towards an integrative view on anuran communication. *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology* 200:777–787. DOI: <https://doi.org/10.1007/s00359-014-0923-1>.
- Starnberger, I., D. Preininger, and W. Hödl. 2014b. The anuran vocal sac: a tool for multimodal signalling. *Animal Behaviour* 97:281–288. DOI: <https://doi.org/10.1016/j.anbehav.2014.07.027>.

- Sztatecsny, M., C. Strondl, A. Baierl, C. Ries, and W. Hödl. 2010. Chin up: are the bright throats of male common frogs a condition-independent visual cue? *Animal Behaviour* 79:779–786.
- Sztatecsny, M., D. Preininger, A. Freudmann, M.-C. Loretto, F. Maier, and W. Hödl. 2012. Don't get the blues: conspicuous nuptial colouration of male moor frogs (*Rana arvalis*) supports visual mate recognition during scramble competition in large breeding aggregations. *Behavioral Ecology and Sociobiology* 66:1587–1593. DOI: <https://doi.org/10.1007/s00265-012-1412-6>.
- Taylor, R.C., and M.J. Ryan. 2013. Interactions of multisensory components perceptually rescue túngara frog mating signals. *Science* 341:273–274. DOI: <https://doi.org/10.1126/science.1237113>.
- Taylor, R.C., B.A. Klein, J. Stein, and M.J. Ryan. 2008. Faux frogs: multimodal signaling and the value of robotics in animal behaviour. *Animal Behaviour* 76:1089–1097.
- Taylor, R.C., B.A. Klein, J. Stein, and M.J. Ryan. 2011. Multimodal signal variation in space and time: how important is matching a signal with its signaler? *The Journal of Experimental Biology* 214:815–820.
- Telford, S.R. 1985. Mechanisms and evolution of inter-male spacing in the painted reed frog (*Hyperolius marmoratus*). *Animal Behaviour* 33:1353–1361. DOI: [https://doi.org/10.1016/S0003-3472\(85\)80198-6](https://doi.org/10.1016/S0003-3472(85)80198-6).
- Toledo, L.F., I.A. Martins, D.P. Bruschi, M.A. Passos, C. Alexandre, and C.F.B. Haddad. 2015. The anuran calling repertoire in the light of social context. *acta ethologica* 18:87–99. DOI: <https://doi.org/10.1007/s10211-014-0194-4>.

Waldman, B., and P.J. Bishop. 2004. Chemical communication in an archaic anuran amphibian. *Behavioral Ecology* 15:88–93.

Wells, K.D. 1988. The Effect of Social Interactions on Anuran Vocal Behavior. Pp. 433–454 in *The Evolution of the Amphibian Auditory System* (B. Fritzsch, W. Wilczynski, M.J. Ryan, T.E. Hetherington, and W. Walkowiak, eds.). John Wiley & Sons, Inc., USA.

Woodley, S.K. 2010. Pheromonal communication in amphibians. *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural and Behavioral Physiology* 196:713–727.

Yovanovich, C., S.M. Koskela, N. Nevala, S.L. Kondrashev, A. Kelber, and K. Donner. 2017. The dual rod system of amphibians supports colour discrimination at the absolute visual threshold. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences* 372:20160066.

Zeyl, J.N., and F. Laberge. 2011. Multisensory signals trigger approach behaviour in the fire-bellied toad *Bombina orientalis*: sex differences and call specificity. *Zoology* 114:369–377. DOI: <https://doi.org/10.1016/j.zool.2011.08.001>.

FIGURES:

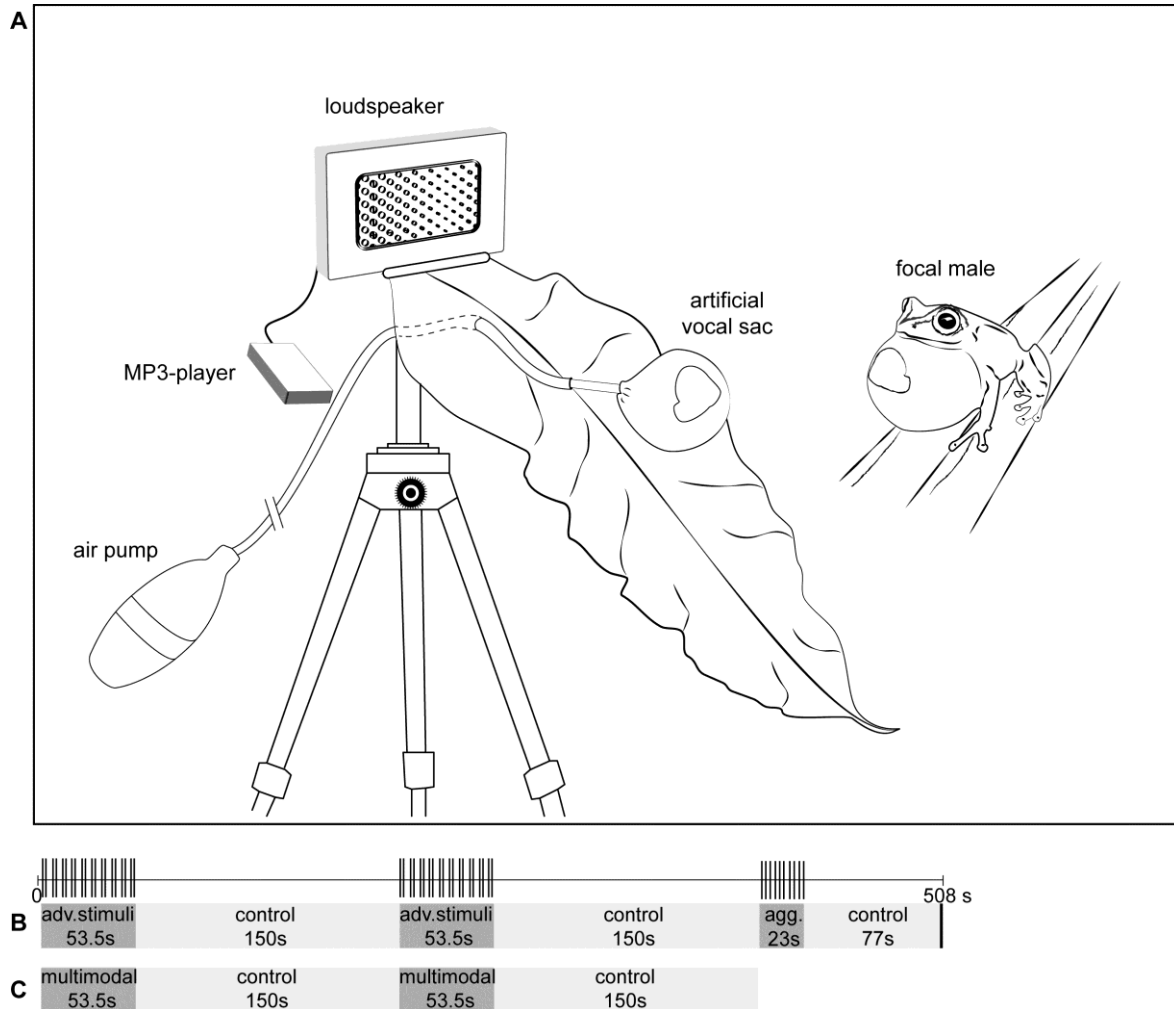


FIG. 1.—Experimental set-up for uni- and multimodal stimulus presentation. Experimental design for uni- and multimodal stimulus presentation. (A) Experimental set-up for playback presentations; (B) schematic of stimulus presentations for acoustic playbacks, black line at the end represents 1 s of white noise; (C) schematic of stimulus presentation for uni- and multimodal presentations, the same randomly selected uni- (acoustic) or multimodal (acoustic and visual) stimuli were presented twice during the period termed "multimodal" to a focal individual.



FIG. 2.—Examples of the diverse dorsal color patterns in male *Hyperolius puncticulatus* in the study population.

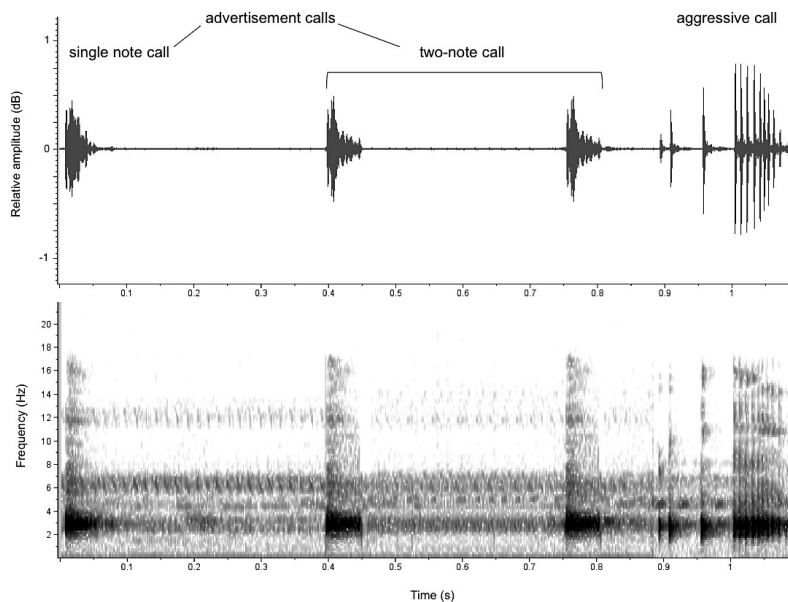


FIG. 3.— Calls of male *Hyperolius puncticulatus*. Waveform and corresponding spectrogram below of two average advertisement calls, with one and two notes and an average aggressive call (80 dB at a 1m distance, 20°C). Inter-call intervals were shortened to present three calls in one graph.

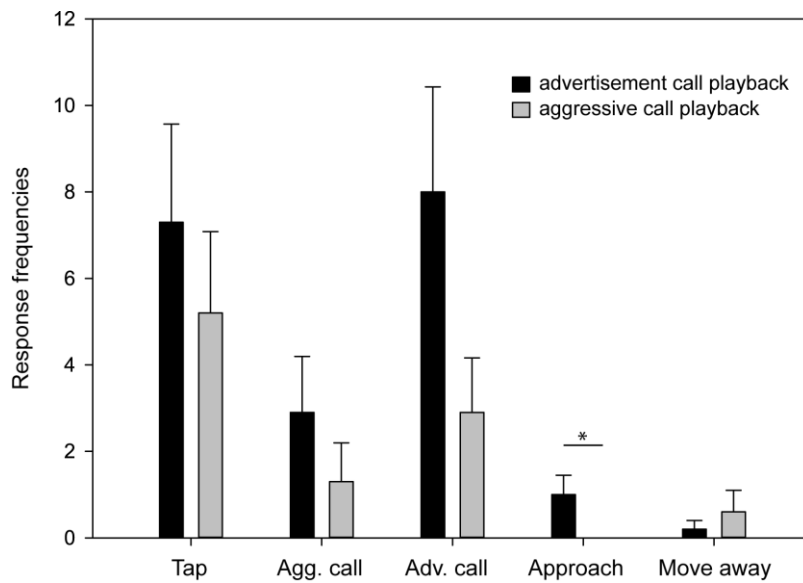


FIG. 4.—Response frequencies to advertisement call and aggressive call play-back. Shown are means and standard error of original response data instead of estimated means. Statistically significant differences between responses are denoted by asterisk (LMMs: * $P < 0.05$; $n = 10$ for all playback presentations)

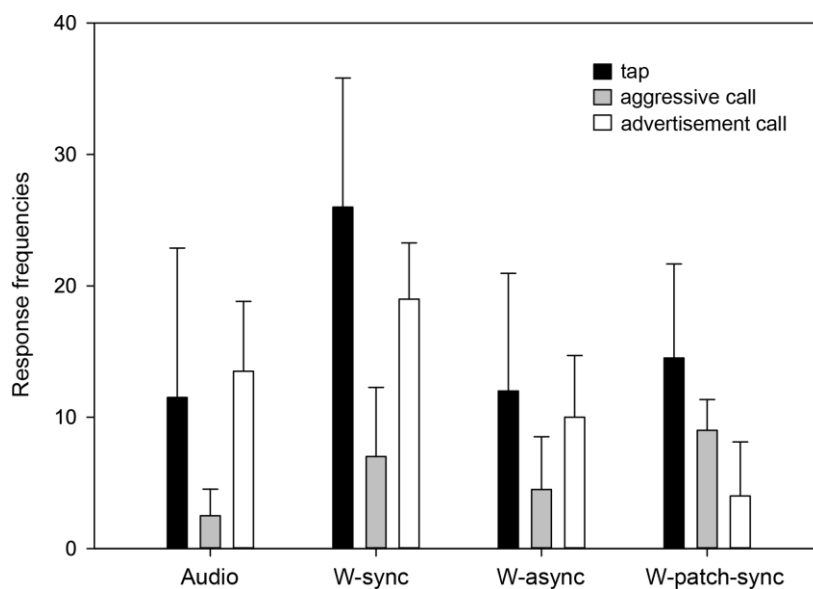


FIG. 5.—Response frequencies to uni- and multimodal stimuli presentations. Stimulus presentations include advertisement call playbacks (audio; $n = 10$), with synchronously moving white vocal sac (W-sync; $n = 11$), with asynchronously moving white vocal sac (W-async; $n = 8$) and with synchronously moving vocal sac with an artificial yellow gular patch (W-patch-sync; $n = 10$).

Concluding Discussion

The studies presented in this thesis investigate the role of the vocal sac in the multimodal signalling system of reed frogs and frogs and toads in general. They address questions about the **mechanism** and the **function** of the gular patch - a characteristic structure found in males of all reed frog species.

In **Chapter 1** we were able to reveal the inner structure of the gular patch which turned out to be a gland complex with ducts leading to the outside of the frog's body. Furthermore, by integrating biochemical analyses, we could identify the components produced in the gland complex. The composition of the chemical cocktail found in gular patch tissue was not only surprisingly complex, but seems to be species-specific at least in the four hyperoliid species investigated. Our results allow drawing conclusions about the **mechanism** of the conspicuous gular patch. However, the evolutionary **function** of producing volatile chemical compounds in a gland complex on the vocal sac is still largely obscure.

In **Chapter 2** we give an overview of several vastly understudied, but potentially highly complex anuran signalling modes, such as foot flagging, dynamic inflation of the vocal sac without the production of a call, or nuptial dichromatism. We argue that combining knowledge about the natural history of a species and about its environment as well as up-to-date methodology will considerably advance our knowledge about anuran communication strategies. Furthermore, we suggest, that anuran communication is more complex than it has been given credit for.

In **Chapter 3** we focused on the anuran vocal sac and investigate its potential as a tool for multimodal signalling. Dynamic cues such as the movement of the vocal sac have long been described as a side-product of call production, because they constitute a fixed-composite signal (*sensu* Partan & Marler, 2005). However, there is increasing evidence that the colouration and/or movement of the vocal sac can act a visual signal in addition to or instead of acoustic signals. Additionally, our findings from **Chapter 1** allowed us to propose that the anuran vocal sac may act as a tool for acoustic, visual and chemical signalling. Accordingly **Chapter 4** highlights the role of acoustic signals with or without additional visual stimuli in male-male communication in a reed frog species. In order to increase our understanding about the adaptive value of the gular patch and the function of the vocal sac in male-male interactions, we tested males' reactions to uni- vs. multimodal signal components produced by an artificial rival male. Surprisingly, we found no differences in responses to unimodal (acoustic) and multimodal (acoustic and visual) signals. Furthermore, the presence of an artificial gular patch on the vocal sac did not elicit different behavioural responses in focal males. However, our field experiments uncovered a previously undescribed signal component: Male Spotted Reed frogs frequently produce **foot taps**

with fore- and hind limbs, which might function in similar context as advertisement calls, to repel males and attract females, but in this case in the visual or seismic domain. Foot tapping might therefore act as a "free" multimodal signal (sensu Higham & Hebets, 2013).

Possible reasons for complex signalling in reed frogs:

The costs of acoustic communication in anurans are relatively high. A calling male is faced with a high energetic expense, an increased risk of predation and a risk of dehydration (Byrne & Keogh, 2007). Females as well as males of many anuran species are confronted with the challenge of locating a particular conspecific male in a **chorus** situation (Bee & Micheyl, 2008; Richardson & Lengagne, 2010) or in **environments** that hamper propagation and transmission of acoustic signals (Boeckle, Preininger, & Hödl, 2009; N. Kime, Turner, & Ryan, 2000; Wells & Schwartz, 1982). Generally, signals should evolve to maximize the visual or acoustic **signal to noise ratio**. This can be accomplished by increasing the amplitude of the signal in a variety of sensory modes (Ryan & Keddy-Hector, 1992) and possibly by using additional and/or multimodal signals. The missing or misinterpretation of a signal is likely related to high costs and a high selective pressure could lead to the use of additional or alternative modalities (Krebs & Dawkins, 1984).

The hyperolid vocal sac is absolutely unique in having the potential to emit acoustic, visual and chemical cues and/or signals. The chemical modality is the one posing the most questions and there are hardly any comparable cases or studies performed in other anuran species (as reviewed in **Chapters 2 and 3**).

I would like to discuss the following hypotheses related to female attraction and female choice in order to explain the adaptive value of the gular gland and its potential chemical signals: A species-specific scent bouquet might **attract females** by facilitating the locatability of a specific male frog and/or by enhancing the attractiveness of male to receptive females (see e.g. Byrne & Keogh, 2007). Furthermore, it might **back-up** information content of the acoustic signal (e.g. species identity, individual health status) or offer **additional information** to an approaching female (Candolin, 2003). Females approaching a multi-species chorus as commonly found in reed frogs (e.g. Starnberger et al., 2013) face the challenge of distinguishing between individual senders - a circumstances which may well select for a complex multimodal display (Hebets & Papaj, 2005).

Another possible explanation might be the need for a **male-male spacing** mechanism mediated by scent signals in a chorus situation in order to minimize energy expenditure due to agonistic

encounters (e.g. Grafe & Thein, 2001). However, after a preliminary experiment performed in the Amani Nature Reserve (Starnberger, unpublished data) I found no evidence to support the latter hypothesis. To investigate, if male *Hyperolius puncticulatus* avoid potential chemical cues left on plant substrate previously used as calling perch by conspecific males, I offered one male frog (n=20) a choice between a twig formerly used as a calling perch (scented twig) and an unscented control twig. I monitored the focal male's position for an hour, but did not find a preference for the scented or the unscented twigs.

I am confident, that **behavioural preference or female choice tests** with receptive females and the scent of live conspecific males or **synthetic frog scent** will shed light on the potential function of the gular patch in intra-specific communication.

The potential **visual signal component** of the gular patch does not seem to be meaningful for male-male interaction at least in *H. puncticulatus*, but once again may be relevant to conspecific females.

Other studies suggest, that animals of many taxa assess multimodal signals in a certain sequence, with **long-range signals** attracting conspecifics for further assessment of **close-range signals** (e.g. Uy & Safran, 2013). Following this suggestion, it is imaginable that receptive females follow the long-range **acoustic signal** of a (multi-species) frog chorus to navigate to the breeding site. Once they have reached the site, they need to find not only a conspecific male within the chorus, but also the male with the most favourable traits. It may well be, that a **pheromone signal** originating from the gular gland complex is a suitable **mid-range** indicator for these properties in addition to the acoustic signal. However, the exact source of both acoustic and chemical signals is rather difficult to locate for a small frog in the dense vegetation. Thus, the **visual stimulus** of the colourful gular patch might considerably facilitate a female's **close-range orientation** towards a male advertising in a patch of reed grass.

Our preliminary studies using spectrophotometry have shown that the hyperolid gular patch clearly stands out from its immediate background - the vocal sac. Both the gular patch and the surrounding vocal sac had a higher reflectance than their environment (i.e. the frog's body as well as the branch the frog is sitting on) under moonlight conditions (Starnberger, unpublished data).

In the case of *Hyperolius puncticulatus* (**Chapter 4**), the additional foot tap which might be perceived in the visual as well as in the seismic domain, could be another way of facilitating close-range orientation towards an advertising male and thereby considerably decreasing a female's search time and predation risk (Grafe, 1997). Furthermore, the foot tap might act as a **back-up**

signal for the advertisement call and may influence both sexes. Since increased locatability most likely represents increased predation risk for the advertising male, could imply that reed frogs not only adjust their overall activity, but vary the use of signal modes in respect to environmental brightness (see Onorati & Vignoli, 2017).

A few thoughts on methodology

This thesis has been laid out as an exploratory, albeit slightly risky taxon-oriented study in order to investigate the intriguing hyperolid gular patch. Due to the multidisciplinary network of collaborators I was able to integrate a diverse set of methods in order to gain a comprehensive overview of the possible function of the gular patch. At the beginning of my PhD studies we already had a strong suspicion that the gular patch might in fact be a gland and thus we concentrated on descriptively investigating this possibility (**Chapter 1**). The next logical step would have been to test the function of the scent bouquets in behavioural experiments. However, the proposed biochemical synthesis turned out to be much more tedious than expected because of the surprising complexity of the chemical cocktails found in the gland. First biochemical results are presented in **Appendix C** and just very recently "Frogolide" a new substance found in the gular glands of *Hyperolius* spp. and the femoral glands of *Mantella* spp. was discovered and will soon be presented in a manuscript by **Menke et al.** (submitted).

Outlook

While the studies comprised in this thesis cover only a small part of the complex hyperolid signalling system, I am confident that my work could serve to inspire future research which will piece by piece add to our understanding of anuran communication.

I would suggest to further **test** the chemical function of single and combined signal components in intra- and intersexual, as well as in intra- and interspecific communication based on **receiver response** as suggested by Higham and Hebets (2013; and see Partan & Marler, 1999; 2005). According my results I would suggest to perform female choice experiments to uni-, bi- and tri-modal signals. This should reveal how multiple signal components increase the signal content, facilitate the perception of each other, or enhance signal transmission (Candolin, 2003; Hebets & Papaj, 2005).

One of the **sensory challenges** for receivers is the cross-modal integration of multimodal stimuli into perceptual objects corresponding to one individual sender (Bee & Miller, 2017). In

frogs in general, but in chorusing frogs in particular, it is important to understand, how receptive females but also rival males manage to **integrate** the often **complex signals** with diverse temporal and spectral parameters.

Furthermore, a thorough investigation of **signal design** might reveal even more **complexity**: For example, how does vocal sac and gular patch colouration as well as scent bouquet composition differ between syntopic species with more similar, compared to species with more different advertisement call parameters? Incorporating increasing complexity as suggested by Hebets (2011) as well as going one step further and investigating individual **signaler plasticity** in a dynamic social and ecological environment (Bro-Jorgensen, 2010; and see Van Staaden & Smith, 2011) will lead to an increased understanding of **signal evolution**. Also, the integration of economic models into the study of advertisement displays in a chorus situation might yield surprising results about the abilities of frogs to dynamically adjust their investment during courtship (Patricelli, Krakauer and McElreath (2011) and about the influence of **adaptive courtship tactics** on individual fitness.

References (of Preface and Concluding Discussion)

- Amézquita, A., & Hödl, W.** (2004). How, when, and where to perform visual displays: the case of the Amazonian frog *Hyla parviceps*. *Herpetologica*, 60(4), 420–429.
- Bee, M. A., & Micheyl, C.** (2008). The cocktail party problem: What is it? How can it be solved? And why should animal behaviorists study it? *Journal of Comparative Psychology*, 122(3), 235–251. <http://doi.org/10.1037/0735-7036.122.3.235>
- Bee, M. A., & Miller, C. T.** (2017). Signaler and Receiver Psychology. In M. A. Bee & C. T. Miller (Eds.), *Psychological Mechanisms in Animal Communication* (Vol. 5, pp. 1–16). Cham: Springer International Publishing. <http://doi.org/10.2173/bna.184>
- Boeckle, M., Preininger, D., & Hödl, W.** (2009). Communication in noisy environments I: acoustic signals of *Staurois latopalmatus* Boulenger 1887. *Herpetologica*, 65(2), 154–165.
- Bradbury, J. W., & Vehrencamp, S. L.** (2011). Signals and Communication. In *Principles of Animal Communication* (2nd ed.). Oxford University Press.
- Bro-Jorgensen, J.** (2010). Dynamics of multiple signalling systems: animal communication in a world in flux. *Trends in Ecology & Evolution*, 25(5), 292–300.
- Butenandt, A., Beckmann, R., & Hecker, E.** (1961). Über den Sexuallockstoff des Seiden-spinners. *Hoppe-Seyler's Zeitschrift Für Physiologische Chemie*, 324(Jahresband), 71–83. <http://doi.org/10.1515/bchm2.1961.324.1.71>
- Byrne, P., & Keogh, J.** (2007). Terrestrial toadlets use chemosignals to recognize conspecifics, locate mates and strategically adjust calling behaviour. *Animal Behaviour*, 74(5), 1155–1162.
- Candolin, U.** (2003). The use of multiple cues in mate choice. *Biological Reviews of the Cambridge Philosophical Society*, 78(4), 575–595. <http://doi.org/10.1017/S1464793103006158>
- Coleman, S.** (2009). Taxonomic and sensory biases in the mate-choice literature: there are far too few studies of chemical and multimodal communication. *Acta Ethologica*, 12(1), 45–48.
- Dawkins, M. S., & Guilford, T.** (1997). Conspicuousness and Diversity in Animal Signals. In D. H. Owings, M. D. Beecher, & N. S. Thompson (Eds.), *Communication. Perspectives in Ethology* (Vol. 12, pp. 55–75). New York: Springer US. http://doi.org/10.1007/978-1-4899-1745-4_3
- De Cock, R., Faust, L., & Lewis, S.** (2014). Courtship and Mating in *Phausis reticulata* (Coleoptera: Lampyridae): Male Flight Behaviors, Female Glow Displays, and Male Attraction to Light Traps. *Florida Entomologist*, 97(4), 1290–1307. <http://doi.org/10.1653/024.097.0404>
- Dinets, V.** (2013). Do individual crocodilians adjust their signaling to habitat structure? *Ethology Ecology & Evolution*, 25(2), 174–184. <http://doi.org/10.1121/1.2714910>
- Drewes, R.** (1984). A phylogenetic analysis of the Hyperoliidae (Anura): treefrogs of Africa, Madagascar, and the Seychelles Islands. *Occasional Papers of the California Academy of Sciences*, 139, 1–70.
- Endler, J. A.** (1991). Variation in the appearance of guppy color patterns to guppies and their predators under different visual conditions. *Vision Research*, 31(3), 587–608. [http://doi.org/10.1016/0042-6989\(91\)90109-I](http://doi.org/10.1016/0042-6989(91)90109-I)

- Farris, H. E., & Taylor, R. C.** (2017). Mate Searching Animals as Model Systems for Understanding Perceptual Grouping. In M. A. Bee & C. T. Miller (Eds.), *Psychological Mechanisms in Animal Communication* (Vol. 5, pp. 89–118). Springer, Cham.
http://doi.org/10.1007/978-3-319-48690-1_4
- Feng, A., Narins, P., & Xu, C.-H.** (2002). Vocal acrobatics in a Chinese frog, *Amolops tormotus*. *Die Naturwissenschaften*, 89(8), 352–356. <http://doi.org/10.1007/s00114-002-0335-x>
- Fitch, W. T.** (2000). The evolution of speech: a comparative review. *Trends in Cognitive Sciences*, 4(7), 258–267. [http://doi.org/10.1016/S1364-6613\(00\)01494-7](http://doi.org/10.1016/S1364-6613(00)01494-7)
- Gaucher, P.** (2002). *Premières données sur Phrynohyas hadroceps, rainette arboricole du plateau des Guyanes (Amphibia: Anura: Hylidae) (Révision taxonomique, Éco-Éthologie de la reproduction)(unpublished Master's Thesis)*. École pratique des hautes études. Sciences de la Vie et de la Terre, Paris, France.
- Gerhardt, H. C., & Schwartz, J. J.** (2001). Auditory tuning and frequency preferences in anurans. In M. J. Ryan (Ed.), *Anuran communicatio* (pp. 73–85). Washington DC: Smithsonian Institution Press.
- Gibson, J. S., & Uetz, G. W.** (2007). Seismic communication and mate choice in wolf spiders: components of male seismic signals and mating success. *Animal Behaviour*, 75(4), 1253–1262. <http://doi.org/10.1016/j.anbehav.2007.09.026>
- Gomez, D., Richardson, C., Lengagne, T., Plenet, S., Joly, P., Léna, J.-P., & Thery, M.** (2009). The role of nocturnal vision in mate choice: females prefer conspicuous males in the European tree frog (*Hyla arborea*). *Proceedings of the Royal Society of London. Series B: Biological Sciences*, 276(1666), 2351–2358.
- Grafe, T. U.** (1997). Costs and benefits of mate choice in the Lek-breeding reed frog, *Hyperolius marmoratus*. *Animal Behaviour*, 53(5), 1103–1117.
- Grafe, T. U., & Thein, J.** (2001). Energetics of calling and metabolic substrate use during prolonged exercise in the European treefrog *Hyla arborea*. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology*, 171(1), 69–76.
<http://doi.org/10.1007/s003600000151>
- Guilford, T., & Dawkins, M. S.** (1991). Receiver psychology and the evolution of animal signals. *Animal Behaviour*, 42(1), 1–14. [http://doi.org/10.1016/S0003-3472\(05\)80600-1](http://doi.org/10.1016/S0003-3472(05)80600-1)
- Hebets, E. A.** (2008). Seismic signal dominance in the multimodal courtship display of the wolf spider *Schizocosa stridulans* Stratton 1991. *Behavioral Ecology*, 19(6), 1250–1257.
<http://doi.org/10.1007/s002650050542>
- Hebets, E. A.** (2011). Current status and future directions of research in complex signaling. *Current Zoology*, 57(2), 1–5.
- Hebets, E. A., & Papaj, D.** (2005). Complex signal function: developing a framework of testable hypotheses. *Behavioral Ecology and Sociobiology*, 57(3), 197–214.
- Higham, J. P., & Hebets, E. A.** (2013). An introduction to multimodal communication. *Behavioral Ecology and Sociobiology*.
- Hopkins, C. D.** (1981). On the diversity of electric signals in a community of Mormyrid electric fish in West Africa. *American Zoologist*, 21(1), 211–222. <http://doi.org/10.1093/icb/21.1.211>

- Kime, N., Turner, W., & Ryan, M. J.** (2000). The transmission of advertisement calls in Central American frogs. *Behavioral Ecology*, 11(1), 71–83.
- Kozak, E. C., & Uetz, G. W.** (2014). Cross-modal integration of multimodal courtship signals in a wolf spider. *Animal Cognition*, 19(6), 1173–1181. doi.org/10.1007/s10071-016-1025-y
- Krebs, J. R., & Dawkins, R.** (1984). Animal signals: mind-reading and manipulation. In J. R. Krebs & N. B. Davies (Eds.), *Behavioural Ecology: An Evolutionary Approach* (2nd ed., Vol. 2, pp. 380–402). Oxford: Blackwell Publishing Ltd.
- Lenoir, A., & Jaisson, P.** (1982). Evolution et rôle des communications antennaires chez les insectes sociaux. In P. Jaisson (Ed.), *Social insects in the tropics* (Vol. 1, pp. 157–180). Paris: Presses de l'Université Paris-Nord.
- Mason, M. J., & Narins, P. M.** (2002). Seismic sensitivity in the desert golden mole *Eremitalpa granti*: A review. *Journal of Comparative Psychology*, 116(2), 158–163.
- Menke, M., Melnik, K., Peram, P. S., Starnberger, I., Hödl, W., Vences, M., & Schulz, S.** (submitted). Frogolide - An unprecedented sesquiterpene macrolactone from scent glands of African frogs. *European Journal of Organic Chemistry*.
- Narins, P. M.** (1990). Seismic communication in anuran amphibians. *Bioscience*, 40(4), 268–274.
- Narins, P. M., Grabul, D. S., Soma, K., Gaucher, P., & Hödl, W.** (2005). Cross-modal integration in a dart-poison frog. *Proceedings of the National Academy of Sciences*, 102(7), 2425–2429.
- Narins, P. M., Hödl, W., & Grabul, D. S.** (2003). Bimodal signal requisite for agonistic behavior in a dart-poison frog, *Epipedobates femoralis*. *Proceedings of the National Academy of Sciences*, 100(2), 577–580.
- Onorati, M., & Vignoli, L.** (2017). The darker the night, the brighter the stars: consequences of nocturnal brightness on amphibian reproduction. *Biological Journal of the Linnean Society*, 120(4), 961–976.
- O'Connell-Rodwell, C. E.** (2007). Keeping an “Ear” to the Ground: Seismic Communication in Elephants. *Physiology*, 22(4), 287–294. http://doi.org/10.1111/j.1365-2664.2005.01044.x
- Papke, R. S., Kemp, D. J., & Rutowski, R. L.** (2007). Multimodal signalling: structural ultraviolet reflectance predicts male mating success better than pheromones in the butterfly *Colias eurytheme* L. (Pieridae). *Animal Behaviour*, 73(1), 47–54. http://doi.org/10.1016/j.anbehav.2006.07.004
- Partan, S. R., & Marler, P.** (1999). Communication goes multimodal. *Science*, 283(5406), 1272–1273.
- Partan, S. R., & Marler, P.** (2005). Issues in the classification of multimodal communication signals. *The American Naturalist*, 166(2), 231–245.
- Patricelli, G. L., Krakauer, A. H., & McElreath, R.** (2011). Assets and tactics in a mating market: Economic models of negotiation offer insights into animal courtship dynamics on the lek. *Current Zoology*, 57(2), 225–236.

- Preininger, D., Boeckle, M., & Hödl, W.** (2009). Communication in noisy environments II: Visual signaling behavior of male foot-flagging frogs *Staurois latopalmatus*. *Herpetologica*, 65(2), 166–173.
- Richardson, C., & Lengagne, T.** (2010). Multiple signals and male spacing affect female preference at cocktail parties in treefrogs. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, 277, 1247–1252.
- Richardson, C., Gomez, D., Durieux, R., Thery, M., Joly, P., Léna, J.-P., Plénet, S. & Lengagne, T.** (2010). Hearing is not necessarily believing in nocturnal anurans. *Biology Letters*, 6(5), 633–635.
- Rödel, M.-O., Kosuch, J., Veith, M., & Ernst, R.** (2003). First record of the genus *Acanthixalus* Laurent, 1944 from the upper Guinean rain forest, West Africa, with the description of a new species. *Journal of Herpetology*, 37(1), 43–52.
- Ryan, M. J., & Keddy-Hector, A.** (1992). Directional patterns of female mate choice and the role of sensory biases. *American Naturalist, Supplement: Sensory Drive*, 139, S4–S35.
- Ryan, M. J., & Kime, N. M.** (2003). Selection on Long-Distance Acoustic Signals. In A. M. Simmons, R. R. Fay, & A. N. Popper (Eds.), *Acoustic Communication* (Vol. 16, pp. 225–274). New York, NY: Springer-Verlag. http://doi.org/10.1007/0-387-22762-8_5
- Sato, S., Tarumizu, K., & Hatae, K.** (1993). The influence of social factors on allogrooming in cows. *Applied Animal Behaviour Science*, 38(3-4), 235–244. [http://doi.org/10.1016/0168-1591\(93\)90022-H](http://doi.org/10.1016/0168-1591(93)90022-H)
- Schiøtz, A.** (1967). The treefrogs (Rhacophoridae) of West Africa. *Spolia Zoologica Musei Hauniensis*, 25, 1–346.
- Shannon, C. E.** (1948). A mathematical theory of communication. *Bell System Technical Journal*, 27, 379–423.
- Starnberger, I., Poth, D., Peram, P. S., Schulz, S., Vences, M., Knudsen, J., Barej, M. F., Rödel, M.-O., Walzl, M. & Hödl, W.** (2013). Take time to smell the frogs: vocal sac glands of reed frogs (Anura: Hyperoliidae) contain species-specific chemical cocktails. *Biological Journal of the Linnean Society*, 110(4), 828–838. <http://doi.org/10.1111/bij.12167>
- Taylor, R. C., Buchanan, B. W., & Doherty, J.** (2007). Sexual selection in the squirrel treefrog *Hyla squirella*: the role of multimodal cue assessment in female choice. *Animal Behaviour*, 74(6), 1753–1763.
- Taylor, R. C., Klein, B. A., & Michael, J.** (2011). Inter-signal interaction and uncertain information in anuran multimodal signals. *Current Zoology*, 57, 153–161.
- Uetz, G. W., Roberts, J. A., Clark, D. L., Gibson, J. S., & Gordon, S. D.** (2013). Multimodal signals increase active space of communication by wolf spiders in a complex litter environment. *Behavioral Ecology and Sociobiology*, 67(9), 1471–1482.
- Uy, J. A. C., & Safran, R. J.** (2013). Variation in the temporal and spatial use of signals and its implications for multimodal communication. *Behavioral Ecology and Sociobiology*, 67(9), 1499–1511. <http://doi.org/10.1007/s00265-013-1492-y>
- Van Staaden, M. J., & Smith, A. R.** (2011). Cutting the Gordian knot: Complex signaling in African cichlids is more than multimodal. *Current Zoology*, 57(2), 237–252.

- Vane-Wright, R. I., & Boppré, M.** (1993). Visual and chemical signalling in butterflies: functional and phylogenetic perspectives. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, 340(1292), 197–205. doi.org/10.1098/rstb.1993.0058
- Vásquez, T., & Pfennig, K.** (2007). Looking on the bright side: females prefer coloration indicative of male size and condition in the sexually dichromatic spadefoot toad, *Scaphiopus couchii*. *Behavioral Ecology and Sociobiology*, 62(1), 127–135.
- Vogt, K., Boos, S., Breitenmoser, U., & Kölliker, M.** (2016). Chemical composition of Eurasian lynx urine conveys information on reproductive state, individual identity, and urine age. *Chemoecology*, 26(6), 205–217. http://doi.org/10.1016/j.anbehav.2016.04.004
- Wahlberg, M., & Larsen, O. N.** (2017). Propagation of sound. In C. Brown & T. Riede (Eds.), *Comparative Bioacoustics: An Overview* (pp. 62–119). Bentham Science Publishers.
- Waldman, B., & Bishop, P. J.** (2004). Chemical communication in an archaic anuran amphibian. *Behavioral Ecology*, 15(1), 88–93.
- Wathan, J., Proops, L., Grounds, K., & McComb, K.** (2016). Horses discriminate between facial expressions of conspecifics. *Scientific Reports*, 6, 38322. doi.org/10.1038/srep38322
- Wells, K. D., & Schwartz, J. J.** (1982). The effect of vegetation on the propagation of calls in the neotropical frog *Centrolenella fleischmanni*. *Herpetologica*, 38(4), 449–455.
- West-Eberhard, M. J.** (2001). The Importance of Taxon-Centered Research in Biology. In M. J. Ryan (Ed.), *Anuran Communication* (1st ed., pp. 3–7). Washington DC: Smithsonian Institution Press.
- Wyatt, T. D.** (2008). Animals in a chemical world. In *Pheromones and Animal Behaviour - Communication by Smell and Taste* (4 ed., p. 12). Cambridge: Cambridge Univ Press.

Appendix

A. Multimodal signaling in the Small Torrent Frog (*Micrixalus saxicola*) in a complex acoustic environment.

Preininger, D., Boeckle, M., Freudmann, A., Starnberger, I., Sztatecsny, M. & W. Hödl.
2013

Publication released: *Behavioral Ecology and Sociobiology*, **67**, 1449–1456.

Contribution: DP, MB, MS designed the experiments. DP, MB performed experiments in the field and collected samples. DP, MB, AF, MS analyzed the data. DP, IS, MS, WH wrote the paper.

B. Take the long way home: Behaviour of a neotropical frog, *Allobates femoralis*, in a detour task

Munteanu, A. M., **Starnberger, I.**, Pašukonis, A., Bugnyar, T., Hödl, W., & Fitch, W. T. 2016
Publication released *Behavioural Processes*, **126**, 71–75.

DOI: 10.1016/j.beproc.2016.03.009

Contribution: IS, AMM, AP, TB, WTF, WH designed the experiments. IS, AMM performed experiments in the field. AMM, IS analyzed the data. AMM, IS, AP, TB, WTF, WH wrote the paper.

C. Identification, Synthesis and Mass Spectrometry of a Macrolide from the African Reed Frog *Hyperolius cinnamomeoventris*.

Menke, M., Peram, P., Starnberger, I., Hödl, W., Jongsma G., Blackburn, D., Rödel, M.-O., Vences, M. & Schulz, S. 2016

Publication released: *Beilstein Journal of Organic Chemistry* 2016, **12**, 2731–2738.
DOI:10.3762/bjoc.12.269

Contribution: MM, PP, MV, SS, designed the experiments. IS, WH, GJ, DB collected samples in the field. MM, PP, SS performed experiments in the lab. MM, PP, SS, analyzed the data. MM, PP, MV, SS wrote the paper.

Behav Ecol Sociobiol (2013) 67:1449–1456
DOI 10.1007/s00265-013-1489-6

ORIGINAL PAPER

Multimodal signaling in the Small Torrent Frog (*Micrixalus saxicola*) in a complex acoustic environment

Doris Preininger · Markus Boeckle · Anita Freudmann ·
Iris Starnberger · Marc Sztatecsny · Walter Hödl

Received: 28 June 2012 / Revised: 10 August 2012 / Accepted: 20 August 2012 / Published online: 13 February 2013
© The Author(s) 2013. This article is published with open access at Springerlink.com

Abstract Many animals use multimodal (both visual and acoustic) components in courtship signals. The acoustic communication of anuran amphibians can be masked by the presence of environmental background noise, and multimodal displays may enhance receiver detection in complex acoustic environments. In the present study, we measured sound pressure levels of concurrently calling males of the Small Torrent Frog (*Micrixalus saxicola*) and used acoustic playbacks and an inflatable balloon mimicking a vocal sac to investigate male responses to controlled unimodal (acoustic) and multimodal (acoustic and visual) dynamic stimuli in the frogs' natural habitat. Our results suggest that abiotic noise of the stream does not constrain signal detection, but males are faced with acoustic interference and masking from conspecific chorus noise. Multimodal stimuli elicited greater response from males and triggered significantly more visual signal responses than unimodal stimuli. We suggest that the vocal sac acts as a visual cue and improves detection and discrimination of acoustic signals by making them more salient to receivers amidst complex biotic background noise.

Keywords Anura · Acoustic signal · Background noise · Multimodal communication · Visual cue · Vocal sac

Introduction

To explain evolutionary patterns in animal communication, it is critical to understand the mechanisms of signal production, the conditions under which signals are produced, and how signals are perceived by receivers (Bradbury and Vehrencamp 2011; Brumm and Slabbekoom 2005; Miller and Bee 2012). During the last decade, it has become clear that communication signals in many taxa are more complex than previously thought (Hebets and Papaj 2005). Complex signals can consist of multiple components in a single modality (e.g., acoustic, visual, or chemical) or in multiple sensory modalities (multimodal communication) with components being presented either together or independently (Partan and Marler 1999; Partan and Marler 2005). In so-called fixed-composite signals (Smith 1977; Partan and Marler 2005), signal components occur always together. Based on their assumed information content, multimodal signals have been classified as redundant (all signal components elicit an equivalent response in the receiver) or non-redundant (signal components elicit a different response in the receiver). Hebets and Papaj (2005) suggested that multiple signal components may evolve when they increase the signal content (content-based hypothesis), facilitate the perception of each other (inter-signal interaction hypothesis), or enhance signal transmission for instance in noisy environments (efficacy-based hypothesis).

Acoustic signal detection and discrimination can be constrained by abiotic and/or biotic noise sources such as waterfalls or vocalizing hetero- or conspecifics, thus favoring the evolution of complex signaling strategies (Gerhardt and Klump 1988; Schwartz and Gerhardt 1989; Brumm and Slabbekoom 2005; Gordon and Uetz 2012), which could facilitate faster and more accurate detection by receivers

Communicated by J. Higham

This manuscript is part of the special issue Multimodal Communication—Guest Editors: James P. Higham and Eileen A. Hebets

D. Preininger (✉) · I. Starnberger · M. Sztatecsny · W. Hödl
Department of Integrative Zoology, University of Vienna,
Althanstraße 14,
1090 Vienna, Austria
e-mail: doris.preininger@univie.ac.at

M. Boeckle
Department of Cognitive Biology, University of Vienna,
Althanstraße 14,
1090 Vienna, Austria

A. Freudmann
Department of Animal Biodiversity, University of Vienna,
Althanstraße 14,
1090 Vienna, Austria

(Rowe 1999; Otovic and Partan 2009). However, the selection pressures influencing signaling strategies may differ when environmental noise originates primarily from conspecifics compared with other types of noise because conspecific noise contains a high degree of frequency and temporal overlap between the signals and noise (Gerhardt and Huber 2002).

Anuran amphibians are excellent model systems to study multimodal communication in noisy environments. In many anurans, males produce loud advertisement calls that mediate both female choice and male spacing (Ryan 2001; Gerhardt and Huber 2002). Frog communication may take place in dense breeding choruses (Bee and Micheyl 2008) and/or noisy settings like streams that produce broadband ambient background noise (Boeckle et al. 2009). While advertising, males inflate and deflate the vocal sac which has a primary evolutionary function of recycling air during vocalization, thereby increasing the call rate and distributing sound waves omnidirectionally (Rand and Dudley 1993; Pauly et al. 2006). Since the vocal sac is inevitably moving while a male is calling, it can send a fixed-composite signal (see Hirschmann and Hödl 2006 for exception) imparting increased detectability due to movement and coloration (Rosenthal et al. 2004; Taylor et al. 2008). For example, in the dart-poison frog *Allobates femoralis*, simultaneous acoustic and visual signals are necessary to evoke an aggressive reaction in males defending a territory (Narins et al. 2003; Narins et al. 2005). For females of the squirrel tree frog (*Hyla squirella*), the availability of the vocal sac as a visual cue makes an unattractive male call more appealing, whereas additional visual information is assessed from lateral body stripes when male calls are equally attractive (Taylor et al. 2007, 2011b). Despite these examples of a preference for multimodal over unimodal signals, there seem to be vast differences in the importance of signal components and the responses they elicit in the receiver, even in species facing similar ecological problems. Since ecological settings to which animals are exposed can be complex (e.g., many calling individuals, various abiotic sources of noise, etc.), we wanted to test receiver responses in a frog's natural habitat by using an experimental model setup. Models present three-dimensional visual stimuli that can be detected from a wide angle of view making their use advantageous when the position of receivers cannot be controlled before starting an experiment. The successful use of robots for testing isolated or combined signal components has been demonstrated in studies on a variety of animals, including frogs (Narins et al. 2003; Taylor et al. 2008; Krause et al. 2011).

The Small Torrent Frog (*Micrixalus saxicola*) occurs along tropical streams and communicates in large social aggregations. Signal detection and discrimination in *M. saxicola* could therefore be constrained by both conspecific chorus noise and ambient stream noise. Males display a bright white vocal sac during vocalizing (Fig. 1) and perform additional visual signals (e.g., foot-flagging) in male–male agonistic interactions (Krishna and



Fig. 1 *M. saxicola* male displaying a bright whitish vocal sac during advertising and foot-flagging behavior

Krishna 2006). Given the acoustically complex environment in which the frogs occur and their signaling behavior, we investigated whether stream noise and/or chorus noise constrains the acoustic signal component in male–male agonistic behaviors (signal efficacy approach, Hebets and Papaj 2005). To do so, we first characterized acoustic features of the male advertisement call and measured sound pressure levels of calls and background noise during the breeding season. Further, we investigated the sensory components in the male display by providing controlled and naturally occurring stimuli (call alone and call with synchronously presented artificial vocal sac) via an experimental setup and examined male responses. These experiments allowed us to test the signal–interaction hypothesis predicting that multimodal composites amplify signal detection and discrimination compared to the unimodal acoustic component (Hebets and Papaj 2005).

Methods

Study site and animals

The Small Torrent Frog (*M. saxicola*) is endemic to the Western Ghats in India (Daniels 2005) and occurs exclusively along small, fast-flowing streams within the evergreen forests (Chandran et al. 2010). Individuals are diurnal and inhabit perennial streams characterized by low water, air, and soil temperature (Reddy et al. 2002). Males produce calls with a series of pulses from exposed sites on rocks in shallow areas of the stream to advertise for females and defend breeding grounds in relatively crowded aggregations (Krishna and Krishna 2006). We studied a population of *M. saxicola* located at the Kathalekan *Myristica* swamp forest (14.27414°N, 74.74704°E) in the central Western Ghats at the end of the monsoon season (September and October 2010). Males in our study population had a mean snout–urostyle length (SUL) of

23.6 mm and a mean mass of 1.1 g ($n=13$). Interindividual distance between calling males was measured to determine average receiver distance. Median distance between advertising individuals in the study population was 0.98 m (range, 0.38–2.69 m, $n=15$).

The frogs were captured with permission of the Centre for Ecological Sciences, Indian Institute of Science, Bangalore (permission number: D.WL.CR-27/2008-09) and released immediately after taking body measurements. All behavioral experiments were performed without physical contact with the study animals.

Acoustic recordings

After locating a vocalizing male, we recorded advertisement calls from a distance of approx. 1 m, using an omnidirectional microphone (Sennheiser Me 62) and a digital recorder (Zoom HN4; settings: 44.1 kHz, 16-bit resolution). We measured peak sound pressure levels with a sound level meter (Votkraft SL-100; settings: fast/max, C-weighted) from a distance of 1 m, which equaled the measured median male interindividual distance of 0.98 m. During recordings, the focal male was closer to the microphone than calling neighbors. One second after each advertisement call, a period of 3 s was selected from omnidirectional call recordings to analyze environmental background noise comprising chorusing conspecifics (termed “chorus noise”). We additionally recorded ambient stream noise without male calls (simply termed as “abiotic noise” despite occasional comprising insect signals) before and after frog choruses from the same recording position as calls. The stream was regarded as noise field in which ambient noise intensity was considered almost unchanging within the measured distance of 1 m. For the call analyses, we discarded recordings with overlapping calls from chorusing males. We measured SUL and body mass of each focal individual after the sound recordings with a sliding caliper to the nearest 0.1 mm and a digital mini scale to the nearest 0.01 g.

Acoustic features of recordings were extracted and measured using custom built programs in PRAAT 5.2.22 DSP package (Boersma and Weenink 2011) that automatically logged relevant variables in an output file. To analyze single call notes, we extracted the voiced intervals of the call and measured note duration in seconds. Call duration in seconds was calculated with note start and end times. The spectral structure of calls was investigated using spectrograms (fast Fourier transform (FFT) method; window length, 0.01; time step, 1,000; frequency step, 500; Gaussian window; and dynamic range, 50 dB). For call frequency analysis, a cross-correlation pitch extraction algorithm was used to produce time-varying numerical representation of the fundamental frequency (F_0) contour for each call. We applied a time step of 0.5 ms over a range of 3,000–6,000 Hz according to the F_0 observed in the spectrogram and extracted the parameters

mean, minimum, and maximum F_0 from the F_0 contour. The mean frequency value ± 500 Hz was used to apply a filter before measuring sound pressure. To extract parameters from noise files, we applied a similar analysis and computed a long-term average spectrum with a bandwidth of 50 Hz to measure maximum frequency. To obtain sound pressure (SP) values of chorus and abiotic noise within the frequency range of the advertisement call, we applied a pass Hann band filter to the spectrum for frequencies from 4,300 to 5,300 Hz. The extracted relative SP values for call and noise were transformed into absolute SP (in pascal) by defining the most intensive SP of the complete sound file (SP absolute = SP relative $\times$ SP measured/SP most intensive). “SP measured” corresponds to the maximum sound pressure recorded in the field.

Playback experiments

Unimodal (acoustic) and multimodal (acoustic and visual) stimuli were presented on a platform made out of two plastic containers (Fig. 2). The larger container ($7 \times 18 \times 11$ cm) was filled with pebbles and placed in the stream where it served as an anchor for the attached smaller container (6×10 cm $\times$ 1 cm) and the loudspeaker (Sony SRS-M 30) connected to an MP3 player (Odys Pax). To test if the vocal sac is the primary visual signal component that makes the display more salient to receivers, we presented males exclusively with an artificial inflatable vocal sac. We did not use a stationary model frog as additional visual stimulus or further identification feature. The tip of a white latex glove (inflated diameter, 1 cm) on top of the small container mimicked the vocal sac, which could be inflated by the experimenter by gently blowing air through a 2.5-m-long hose. We tested 10

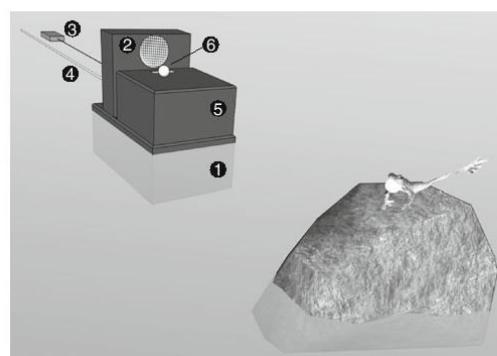


Fig. 2 Schematic diagram of the experimental setup positioned 50 cm from the focal individual. In the stream, the lower box (1) serves as anchor to the upper setup and a loudspeaker (2) connected to an MP3 player (3). Silicon hose (4) operated by the experimenter inserted through the upper box (5) to the artificial vocal sac: tip of a latex glove (6)

males with unimodal playbacks and 10 males with multimodal playback presentations resulting in a total of 20 tested individuals (identifiable through photos). Playback stimuli were presented from the experimental setup, placed 50 cm from the focal individual. From a distance of 1 m from the focal male, the experimenter operated the MP3 player and in multimodal presentation manually inflated the artificial vocal sac synchronously with each call. The prerecorded advertisement call was generated by using averaged call values from the studied population (call duration, 2.6 s; note number, 21; mean frequency, 4.6 kHz; intercall interval, 7.4 s). The acoustic stimulus consisted of three advertisement calls with an average intensity of 75 dB at 50 cm. Experimental playback presentations were undertaken only when the focal individual showed no signaling behavior in the prior 60 s. Either unimodal or multimodal stimuli were presented for a period of 30 s, followed by a 90-s control phase and a subsequent second same stimulus playback and control phase. All trials were video recorded with a waterproof camera (Sanyo Xacti WH1) positioned on a tripod. We analyzed frequencies and durations of the behavior categories “calling,” “tapping,” “foot-flagging,” and “position change” during presentation and control phases with the behavioral coding software Solomon Coder (Péter 2011). Tapping constitutes the lifting of either the right or left leg without stretching it, whereas foot-flagging labels the behavior of completely extending the leg above and back in an arc and bringing it back to the body side (Hödl and Amézquita 2001). Behaviors termed position change included approach, moving away, and turn. Digital photographs of dorsal patterns and colorations allowed individual recognition and ensured that we were able to avoid repeat testing of the same individuals.

Data analysis

To test the hypothesis that *M. saxicola* advertisement calls are masked by noise, we analyzed 112 calls and respective noise recordings from 13 individuals, in turn comprising measurements at 13 positions in the stream. We compared maximum sound pressure values of the acoustic factors: advertisement calls, abiotic noise, abiotic noise in the frequency range of the call (filtered abiotic noise), and chorus noise in the frequency range of the call (filtered chorus noise) using a linear mixed model (LMM). The LMM allows repeated measurements of the same individual to be fitted in the model as random variables, thus controlling for differing number of calls per male and notes per call. The statistical assumptions for LMM analysis were met (Kolmogorov–Smirnov test).

The sound pressure values (in pascal) of all acoustic factors were entered as dependent variables, with the acoustic factors as predictor variables. We entered the identities of male (call) and call (note) as nested random variables, to correct for differences between male individuals, number of calls per

male and number of notes per call. For post hoc tests, we used Student's *t* statistic with sequential Bonferroni correction for alpha because of repeated pairwise comparisons.

A second LMM was conducted to evaluate the differences between dominant frequencies of call and background noise. To compare frequencies of call and noise, the dominant frequencies of these parameters were entered as dependent variables with call and noise as predictor variables. The identities of male (call) and call (note) were entered as nested random variables. To test if male SUL and body mass influence mean dominant call frequency, we performed a linear regression analysis.

To investigate the hypothesis that the inflating vocal sac acts as an additional visual cue, we compared behavioral responses to unimodal and multimodal playbacks using a two-tailed Mann–Whitney *U* test for independent samples. To analyze differences in call duration in response to playback presentations, we used a LMM to correct for differing numbers of calls per individual. Call duration was entered as a dependant variable, with modality (unimodal vs. multimodal) as the predictor variable. A nested term was included for the identity of male (call) as a random variable. All analyses were undertaken using SPSS version 19 (SPSS Inc., Chicago, IL, USA).

Results

Frog calls and environmental noise

Advertisement calls of *M. saxicola* (Fig. 3) had an average duration of 2.0 ± 0.1 s (all results $\pm$ SE and $N=13$ in all cases) and comprised a series of 21 ± 1 notes with an average duration of 0.021 ± 0.001 s (Fig. 3a, b). Single pulsed notes were produced at the beginning and end of the call with an inter-note interval of 0.136 ± 0.005 s, whereas grouped notes in the middle of the call had multiple pulses and an inter-grouped notes interval of 0.03 ± 0.001 s (Fig. 3c). The frequency of the advertisement calls averaged $4,771 \pm 29$ Hz (range, 4,574–4,969 Hz, Fig. 3d) and was negatively influenced by SUL (linear regression: $N=13$, $r^2=0.37$, $P=0.016$), but not affected by body weight (linear regression: $N=13$, $r^2=0.08$, $P=0.176$).

The call frequency showed clear differences to the low-frequency dominated stream noise (LMM: pairwise comparison, $\beta=4168$; SE=22; $t=188.087$, $P<0.001$). The maximum SP of the call averaged 0.056 Pa (69 dB) at a distance of 1 m. Overall SP comparisons of call and noise differed significantly (LMM: $F_{3, 2,357}=39.806$, $P<0.001$). At 1 m distance, the call had a higher SP than abiotic noise values (LMM: pairwise comparison, $P<0.001$, Fig. 4), but did not differ from the SP of conspecific chorus noise filtered in the frequency range of the call (LMM: pairwise comparison, $\beta=0.010$; SE=0.005; $t=1.829$, $P=0.068$). The

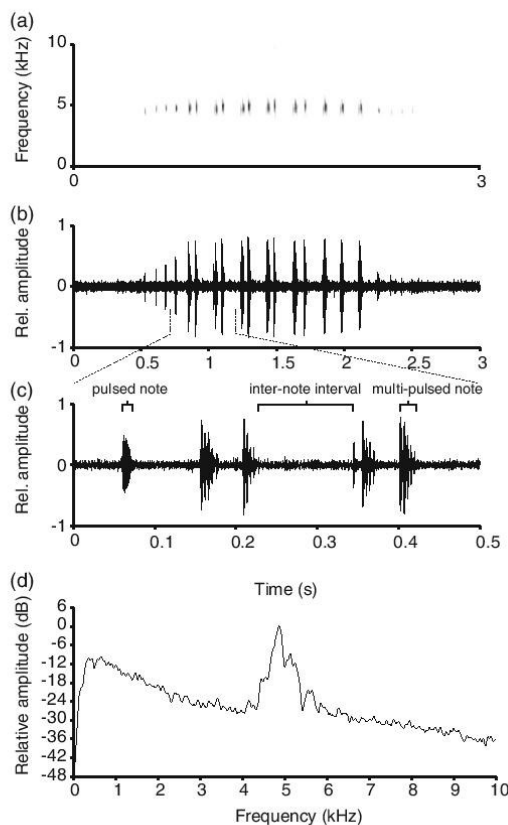


Fig. 3 Multi-note advertisement call of *M. saxicola* (a–d). The spectrogram (a) of a single call (FFT method; window length, 0.005 s; time step, 1,000; frequency step, 1,000; Gaussian window; dynamic range, 30 dB); b the corresponding waveform; c a close-up of the three indicated notes from the same male; d power spectrum showing the peak of the call at 4.9 kHz relative to the ambient stream noise

estimated maximum SP of chorus noise averaged 0.046 Pa (67 dB) resulting in a difference of –2 dB relative to the analyzed frog calls.

Playback experiments

When presented with multimodal stimuli, all tested males increased the number of calls they produced (Mann–Whitney U test=12, $N_1=N_2=10$, $P=0.004$) and tapping behaviors (Mann–Whitney U test=12, $N_1=N_2=10$, $P=0.002$) and performed more position changes (Mann–Whitney U test=17, $N_1=N_2=10$, $P=0.007$) in comparison to unimodal trials. Most interestingly, foot-flagging behavior could only be elicited by multimodal playbacks (Fig. 5) and the mean call duration of 2.2 ± 0.7 s during unimodal playbacks expanded to 5.0 ± 0.36 s

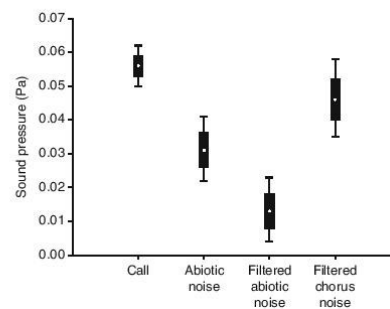


Fig. 4 Comparison of sound pressure of advertisement calls of *M. saxicola* and the background noise. Shown here are estimated means (points), standard error (boxes), and 95 % confidence intervals (whiskers) of the call ($N=13$), abiotic noise ($N=13$), abiotic noise filtered in the frequency range (4.3–5.3 kHz) of the call ($N=13$), and chorus noise filtered in the same frequency range as abiotic noise. All pairwise comparisons apart from call and chorus noise filtered indicate significant differences ($P<0.001$)

during multimodal presentations (LMM: $F_{1, 165}=12.519$, $P=0.001$, Fig. 6).

Discussion

Our results show that sound pressure levels of *M. saxicola* male calls significantly exceeded the ambient abiotic noise level in the frogs' habitat. Stream noise had less energy than frog calls across the entire human audible frequency range. Contrary to species of the Bornean Splash Frogs (*Staurois*) who inhabit low-frequency dominated, torrential streams and waterfalls (Boeckle et al. 2009; Grafe et al. 2012), continuous stream noise alone was unlikely to constrain acoustic signal detection in *M. saxicola*. Chorus noise, however, appeared more likely to hamper individual call detection as the measured SPL differences between male *M. saxicola* calls and

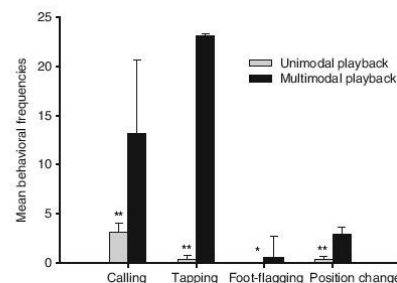


Fig. 5 Behavioral responses of *M. saxicola* to unimodal and multimodal playback stimuli. ** $P<0.01$; * $P<0.05$, statistical significant differences between responses (Mann–Whitney U test; for each stimulus presentation $N=10$)

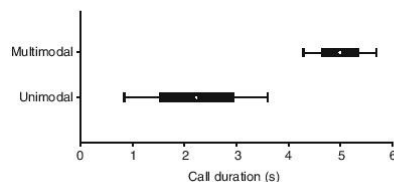


Fig. 6 Differences in duration of *M. saxicola* calls in response to acoustic- and multimodal playback presentations ($P=0.001$; LMM)

chorus noise were small (2 dB). Unfortunately, the perceptual capabilities of *M. saxicola*'s auditory system are unknown but studies on other frog species suggests that the substantial frequency overlap of conspecific noise observed in the present study is likely to interfere with acoustic signal detection (Wilczynski et al. 1993; Schwartz and Gerhardt 1998; Woller and Wiley 2002; Bee 2008; also see Schwartz and Gerhardt 1998 for improved call detection in the presence of noise). Chorus noise can vary based on seasonal and population density thereby creating a fluctuating environment, which has been suggested to favor the evolution of multimodal signals (Bro-Jørgensen 2010).

In the presence of noise, several different strategies can increase the probability of signal recognition and detection. One strategy to provide release from masking is spatial distribution. In Cope's gray tree frog (*Hyla chrysoscelis*), improvements in signal detection and mate recognition are obtained when a signal is spatially separated from a masker, particularly at a signal-to-noise ratio (SNR) of -3 dB (Bee 2008). However, if calls are not spatially separated from background noise, females of a Neotropical treefrog (*Hyla ebraccata*) located calls with $+3$ dB but not $+1.5$ dB SNR (Woller and Wiley 1999). In several bird species, spectral shifts and/or amplitude adjustments have been reported in areas with noise (Slabbekoorn and Peet 2003; Nemeth and Brumm 2010), likewise adaptive strategies such as high-frequency calls have evolved in anurans (Feng and Narins 2008; Boeckle et al. 2009) to increase the signal-to-noise ratio in unimodal signals. There are also an increasing number of examples of the use of visual displays to enhance signal efficacy in noisy environments including in crabs (*Uca mjoebergi*) and lizards (*Anolis cristatellus*, *A. gundlachi*, and *Amphibolurus muricatus*) (Peters and Evans 2003; Ord et al. 2007; Milner et al. 2008).

The observed $+2$ dB SNR in *M. saxicola* could be a sufficient detection threshold, but we also have to consider that the results can be explained by differences in distance to the microphone between the focal male and the more distant, and thereby degraded, neighbor calls. In dense aggregations and close-range interactions, we would expect the SNR to be less or even negative depending on the position of the receiver. Hence, spatial segregation of opponent males could reduce

masking, and interacting signal components could be beneficial for early detection and localization of conspecifics.

In our behavioral experiments, multimodal stimuli significantly increased the frequency of response behaviors compared to unimodal acoustic stimuli, and foot-flagging behavior could only be elicited by multimodal stimuli. We suggest that the visual component acts as an amplifier to the acoustic component supporting the inter-signal interaction hypothesis. The advertisement call may serve as long-range signal (Bee 2007), and integration of a pulsating vocal sac could facilitate localization in dense aggregations of concurrently calling individuals (Gomez et al. 2011; Taylor et al. 2011a, b). The localization and detection of a caller is more difficult when masked by conspecific calls with a high degree of spectral overlap (Marshall et al. 2006), making the visual epiphenomenon particularly advantageous in large choruses. The visual component as part of the acoustic signal has been suggested to modulate male reactions including attacks (Narins et al. 2003; de Luna et al. 2010); therefore, multimodal signals displayed in close proximity could trigger a more intense response. Across-species quantification of multimodal signals in spiders (Hebets 2008), fish (Van Staaden and Smith 2011), and anurans (Taylor et al. 2011a) provide evidence for response variations and highlight differences in signal dominance and receiver perception. It remains difficult to draw assumptions on signal function, but quantification of receiver responses under different environmental conditions will help to explain the processes acting on complex signals.

Hödl and Amézquita (2001) discussed ecological conditions favoring the evolution of visual signals in anurans such as displays at elevated perches, diurnality, and ambient noise which all apply to the study species *M. saxicola*. Although males can be observed advertising around the year, aggregation density is greatest during the presumable main breeding period at the end of the monsoon season (Gururaja, personal communication). During this period, large aggregations form in certain parts of the stream that provide favorable conditions for reproduction, such as shallow water riffle areas where males perch on rocks and display and females dig underwater oviposition cavities (Gururaja 2010).

When attending conspecific choruses, males increase the probability of attracting a mate (Gerhardt and Huber 2002) but have to face continuous noise levels and limited options to deal with masking interference. However, an increase in signal duration as observed in *M. saxicola* males during multimodal stimuli presentations could not only indicate fighting ability to the opponent and facilitate spacing but enhance a male's detectability for females in a chorus. Additionally, playback experiments in numerous anuran species have demonstrated that females prefer long call durations usually associated with energetic costs (Gerhardt and Huber 2002) and possible weight loss in males during

the breeding season (Murphy 1994). Calling activity and mating tactics could be related to body mass in *M. saxicola*, whereas spectral features of the call, determined mostly by larynx size (Gerhardt and Huber 2002), are not expected to be affected by the weight of an individual. Snout-urostyle length, however, showed a negative influence on call frequency, which could be a reliable cue to body size for receivers when detecting a call. Lower frequency calls would indicate larger body size usually preferred by female conspecifics (Ryan and Keddy-Hector 1992).

We conclude that limited shallow water areas in the stream used for reproduction by *M. saxicola* lead to strong competition between males, and dense breeding choruses create constant background noise levels. Our results indicate that multimodal signals are necessary to evoke agonistic behavior in this species. Thus, we suggest that the acoustic signal component modulated by the visual component makes the display more salient and facilitates localization and detection of nearby opponent individuals.

The vocal sac in anurans did not evolve as a visual cue but as an organ to improve calling ability, yet its role in communication has been demonstrated in a number of studies. Due to its evolutionary background, the vocal sac's secondary function as a visual cue or signal component is inevitably linked to the acoustic component. Accordingly, it seems not surprising that the present and several other studies found interaction between the acoustic and visual signal components (Narins et al. 2003; Rosenthal et al. 2004; Gomez et al. 2011; Zeyl and Laberge 2011). In the majority of anuran species, the vocal sac is a multimodal fixed-composite signal, but demonstrating whether the visual component adds additional signal content not included in the call and assessing signal information content remain difficult tasks. In *M. saxicola*, the visual displays (e.g., foot-flagging) presented independently of calls further add to the complexity in communication behavior but may also allow for sophisticated behavioral experiments. We suggest a research approach focusing on receiver detection sensitivity and receiver response to visual signaling behaviors that can be performed independently of the auditory signal (e.g., foot-flagging) to explain how selection on senders and receivers promotes complex displays under different acoustic and environmental conditions. Moreover, further across-species comparisons of how single and combined signal components influence receivers are essential to draw conclusions on signal function.

Acknowledgments We thank K. V. Gururaja, S. P. Vijayakumar, and V. Torsekar for their logistic and professional help at the study site and K. Shanker for his scientific collaboration. B. Weissinger helped analyzing video recordings. A. T. Hedge and his family provided us a pleasant stay. We thank V. Arch, R.C. Taylor, and an anonymous reviewer for very helpful comments on the manuscript. The study was supported by the Austrian Science Fund FWF-P22069 and the University of Vienna FS 100/2012.

Ethical standards All experiments reported in this article comply with the current laws of the country in which they were performed.

Conflict of interest The authors declare that they have no conflict of interest.

Open Access This article is distributed under the terms of the Creative Commons Attribution License which permits any use, distribution, and reproduction in any medium, provided the original author(s) and the source are credited.

References

- Bee MA (2007) Sound source segregation in grey treefrogs: spatial release from masking by the sound of a chorus. *Anim Behav* 74:549–558
- Bee MA (2008) Finding a mate at a cocktail party: spatial release from masking improves acoustic mate recognition in grey treefrogs. *Anim Behav* 75:1781–1791
- Bee MA, Micheyl C (2008) The cocktail party problem: what is it? How can it be solved? And why should animal behaviorists study it? *J Comp Psychol* 122:235–251
- Boeckle M, Preininger D, Hödl W (2009) Communication in noisy environments I: acoustic signals of *Staurois latopalmatus* Boulenger 1887. *Herpetologica* 65:154–165
- Boersma P, Weenink D (2011) PRAAT: doing phonetics by computer (Version 5.2.22) [Computer program]. Retrieved Feb 1, 2011, from <http://www.praat.org>
- Bradbury JW, Vehrencamp SL (2011) Principles of animal communication, Second Edition. Sinauer Press, Sunderland
- Bro-Jørgensen J (2010) Dynamics of multiple signalling systems: animal communication in a world in flux. *Trends Ecol Evol* 25:292–300
- Brumm H, Slabbekoorn H (2005) Acoustic communication in noise. *Adv Study Behav* 35:151–209
- Chandran MDS, Rao GR, Gururaja KV, Ramachandra TV (2010) Ecology of the swampy relic forests of Kathalekan from Central Western Ghats. *Bioremed Biodiv Bioavail* 4:54–68
- Daniels RJR (2005) Amphibians of Peninsular India. University Press (India), Hyderabad
- de Luna AG, Hödl W, Amézquita A (2010) Colour, size and movement as visual subcomponents in multimodal communication by the frog *Allobates femoralis*. *Anim Behav* 79:739–745
- Feng AS, Narins PM (2008) Ultrasonic communication in concave-eared torrent frogs (*Amolops tormotus*). *J Comp Physiol A* 194:159–167
- Gerhardt HC, Huber F (2002) Acoustic communication in insects and anurans: common problems and diverse solutions. University of Chicago Press, Chicago
- Gerhardt HC, Klump GM (1988) Masking of acoustic signals by the chorus background noise in the green tree frog: a limitation on mate choice. *Anim Behav* 36:1247–1249
- Gomez D, Théry M, Gauthier A-L, Lengagne T (2011) Costly help of audiovisual bimodality for female mate choice in a nocturnal anuran (*Hyla arborea*). *Behav Ecol* 22:889–898
- Gordon SD, Uetz GW (2012) Environmental interference: impact of acoustic noise on seismic communication and mating success. *Behav Ecol* 23:707–714
- Grafte TU, Preininger D, Szatcscny M, Kasah R, Dehling JM, Proksch S, Hödl W (2012) Multimodal communication in a noisy environment: a case study of the Bornean rock frog *Staurois parvus*. *PLoS One* 7:e37965
- Gururaja KV (2010) Novel reproductive mode in a torrent frog *Micrixalus saxicola* (Jerdon) from the Western Ghats, India. *Zootaxa* 2642:45–52

- Hebets EA (2008) Seismic signal dominance in the multimodal courtship display of the wolf spider *Schizocosa stridulans* Stratton 1991. *Behav Ecol* 19:1250–1257
- Hebets EA, Papaj DR (2005) Complex signal function: developing a framework of testable hypotheses. *Behav Ecol Sociobiol* 57:197–214
- Hirschmann W, Hödl W (2006) Visual signaling in *Phrynobatrachus krefftii* Boulenger, 1909 (Anura: Ranidae). *Herpetologica* 62:18–27
- Hödl W, Amézquita A (2001) Visual signaling in anuran amphibians. In: Ryan MJ (ed) *Anuran communication*. Smithsonian Institution Press, Washington, DC, pp 121–141
- Krause J, Winfield AFT, Deneubourg J-L (2011) Interactive robots in experimental biology. *Trends Ecol Evol* 26:369–375
- Krishna SN, Krishna SB (2006) Visual and acoustic communication in an endemic stream frog, *Micrixalus saxicolus* in the Western Ghats, India. *Amphibia-Reptilia* 27:143–147
- Marshall VT, Schwartz JJ, Gerhardt HC (2006) Effects of heterospecific call overlap on the phonotactic behaviour of grey treefrogs. *Anim Behav* 72:449–459
- Miller CT, Bee MA (2012) Receiver psychology turns 20: is it time for a broader approach? *Anim Behav* 83:331–343
- Milner RNC, Jennions MD, Backwell PRY (2008) Does the environmental context of a signalling male influence his attractiveness? *Anim Behav* 76:1565–1570
- Murphy CG (1994) Determinants of chorus tenure in barking treefrogs (*Hyla gratiosa*). *Behav Ecol Sociobiol* 34:285–294
- Narins PM, Hödl W, Grabul DS (2003) Bimodal signal requisite for agonistic behavior in a dart-poison frog, *Epipedobates femoralis*. *Proc Natl Acad Sci USA* 100:577–580
- Narins PM, Grabul DS, Soma KK, Gaucher P, Hödl W (2005) Cross-modal integration in a dart-poison frog. *Proc Natl Acad Sci USA* 102:2425–2429
- Nemeth E, Brumm H (2010) Birds and anthropogenic noise: are urban songs adaptive? *Am Nat* 176:465–475
- Ord TJ, Peters RA, Clucas B, Stamps JA (2007) Lizards speed up visual displays in noisy motion habitats. *Proc R Soc Lond B: Biol Sci* 274:1057–1062
- Otovic P, Partan S (2009) Multimodal signaling in animals. In: Squire LR (ed) *Encyclopedia of neuroscience*. Academic, Oxford, pp 1095–1105
- Partan S, Marler P (1999) Communication goes multimodal. *Science* 283(5406):1272–1273
- Partan S, Marler P (2005) Issues in the classification of multimodal communication signals. *Am Nat* 166(2):231–245
- Pauly GB, Bernal XE, Rand AS, Ryan MJ (2006) The vocal sac increases call rate in the Túngara frog *Physalaemus pustulosus*. *Physiol Biochem Zool* 79:708–719
- Péter A (2011) Solomon Coder (version beta 11.01.22): a simple solution for behavior coding. <http://solomoncoder.com/>
- Peters RA, Evans CS (2003) Design of the Jacky dragon visual display: signal and noise characteristics in a complex moving environment. *J Comp Physiol A* 189:447–459
- Rand AS, Dudley R (1993) Frogs in helium: the anuran vocal sac is not a cavity resonator. *Physiol Zool* 66:793–806
- Reddy AH, Gururaja KV, Krishnamurthy SV (2002) Habitat features of an endemic anuran *Micrixalus saxicola* Jerdon, 1853 (Amphibia: Ranidae) in central Western Ghats, India. *Amphibia-Reptilia* 23:370–374
- Rosenthal GG, Rand AS, Ryan MJ (2004) The vocal sac as a visual cue in anuran communication: an experimental analysis using video playback. *Anim Behav* 68:55–58
- Rowe C (1999) Receiver psychology and the evolution of multicomponent signals. *Anim Behav* 58:921–931
- Ryan MJ (2001) *Anuran communication*. Smithsonian Institution Press, Washington, DC
- Ryan MJ, Keddy-Hector A (1992) Directional patterns of female mate choice and the role of sensory biases. *Am Nat* 139:4–35
- Schwartz JJ, Gerhardt HC (1989) Spatially mediated release from auditory masking in an anuran amphibian. *J Comp Physiol A* 166:37–41
- Schwartz JJ, Gerhardt HC (1998) The neuroethology of frequency preferences in the spring peeper. *Anim Behav* 56:55–69
- Slabbekoorn H, Peet M (2003) Birds sing at a higher pitch in urban noise. *Nature* 424:267
- Smith WJ (1977) *The behavior of communicating: an Ethological Approach*. Harvard University Press, Cambridge, MA
- Taylor RC, Buchanan BW, Doherty JL (2007) Sexual selection in the squirrel treefrog *Hyla squirella*: the role of multimodal cue assessment in female choice. *Anim Behav* 74:1753–1763
- Taylor RC, Klein BA, Stein J, Ryan MJ (2008) Faux frogs: multimodal signalling and the value of robotics in animal behaviour. *Anim Behav* 76:1089
- Taylor RC, Klein BA, Ryan MJ (2011a) Inter-signal interaction and uncertain information in anuran multimodal signals. *Curr Zool* 57:153–161
- Taylor RC, Klein BA, Stein J, Ryan MJ (2011b) Multimodal signal variation in space and time: how important is matching a signal with its signaler? *J Exp Biol* 214:815–820
- Van Staaden MJ, Smith AT (2011) Cutting the Gordian knot: complex signaling in African cichlids is more than multimodal. *Curr Zool* 57:237–251
- Wilczynski W, McClelland BE, Rand AS (1993) Acoustic, auditory, and morphological divergence in three species of Neotropical frog. *J Comp Physiol A* 172:425–438
- Wollerman L (1999) Acoustic interference limits call detection in a Neotropical frog *Hyla ebraccata*. *Anim Behav* 57:529–536
- Wollerman L, Wiley RH (2002) Background noise from a natural chorus alters female discrimination of male calls in a Neotropical frog. *Anim Behav* 63:15–22
- Zeyl JN, Laberge F (2011) Multisensory signals trigger approach behaviour in the fire-bellied toad *Bombina orientalis*: sex differences and call specificity. *Zoology* 114:369–377



Contents lists available at ScienceDirect

Behavioural Processes

journal homepage: www.elsevier.com/locate/behavprocTake the long way home: Behaviour of a neotropical frog, *Allobates femoralis*, in a detour taskAlexandru Marian Munteanu^{a,*}, Iris Starnberger^b, Andrius Pašukonis^a, Thomas Bugnyar^a, Walter Hödl^b, William Tecumseh Fitch^a^a Department of Cognitive Biology, University of Vienna, Althanstrasse 14, A-1090 Vienna, Austria^b Department of Integrative Zoology, University of Vienna, Althanstrasse 14, A-1090 Vienna, Austria

ARTICLE INFO

Article history:

Received 15 January 2016

Received in revised form 14 March 2016

Accepted 16 March 2016

Available online 17 March 2016

Keywords:

Amphibian behaviour

Spatial cognitive flexibility

Dendrobatidae

Homing

Obstacle avoidance

ABSTRACT

Detour behaviour, an individual's ability to reach its goal by taking an indirect route, has been used to test spatial cognitive abilities across a variety of taxa. Although many amphibians show a strong homing ability, there is currently little evidence of amphibian spatial cognitive flexibility. We tested whether a territorial frog, *Allobates femoralis*, can flexibly adjust its homing path when faced with an obstacle. We displaced male frogs from their calling sites into the centre of circular arenas and recorded their escape routes. In the first experiment we provided an arena with equally high walls. In the second experiment we doubled the height of the homeward facing wall. Finally, we provided a tube as a shortcut through the high wall. In the equal-height arena, most frogs chose to escape via the quadrant facing their former calling site. However, when challenged with different heights, nearly all frogs chose the low wall, directing their movements away from the calling site. In the "escape tunnel" experiment most frogs still chose the low wall. Our results show that displaced *A. femoralis* males can flexibly adjust their homing path and avoid (presumably energetically costly) obstacles, providing experimental evidence of spatial cognitive flexibility in an amphibian.

© 2016 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

Survival and reproductive success depend on an animal's ability to efficiently find its way through the habitat towards resources such as food, water, mating partners and shelter. Most animals live in complex habitats containing irregular terrain and structures that block the direct path. An animal must be able to flexibly overcome such obstacles in order to reach its objectives (Kohlsdorf and Biewener, 2006), even if that involves increasing its distance from, or giving up direct sight of, the goal (Tarsitano, 2006; Zucca et al., 2005).

Detour behaviour studies were pioneered by Köhler (1999) who showed that chimpanzees, dogs and chicks could reach their target by going around physical barriers. Detour studies have since been used to study behavioural plasticity in other mammals (Baragli et al., 2010; Chapuis and Varlet, 1987; Chapuis et al., 1983; Kimchi and Terkel, 2003; Nesterova and Hansen, 2009; Poucet et al., 1983; Smith and Litchfield, 2010; Wynne and Leguet, 2004), birds

(Regolin et al., 1995, 1994; Zucca et al., 2005), reptiles (Lustig et al., 2013), arachnids (Tarsitano and Jackson, 1994, 1997; Tarsitano, 2006) and insects (Collett, 1996). Most detour tests are performed in artificial laboratory conditions and test an animal's ability to detour when the goal is represented by food (Regolin et al., 1995; Tarsitano and Jackson, 1994; Tarsitano, 2006; Wynne and Leguet, 2004) or social reward (Bisazza et al., 1997; Regolin et al., 1995; Zucca et al., 2005). Many amphibians naturally show long distance goal directed movements to breeding or home sites (Sinsch, 2014). Although the sensory basis of spatial orientation has been studied in several anuran species, very little is known about the flexibility involved in their way-finding (but see Collett, 1982; Ingle, 1973, 1970; Lock and Collett, 1980, 1979). Green toads (*Pseudepidalea viridis*) are able to take a detour around a semi-transparent fence to reach food, but apparently are unable to compare different path lengths, and take routes even if they have a visible obstacle further along the path (Collett, 1982).

Poison frogs (Dendrobatidae) display some of the most complex spatial behaviours known among amphibians, such as territoriality and tadpole transport. The brilliant-thighed poison frog (*Allobates femoralis*) is a small leaf litter species common throughout the Amazon basin and the Guiana Shield (Amézquita et al., 2009).

* Corresponding author at: Department of Cognitive Biology, University of Vienna Althanstrasse 14, 1091 Vienna, Austria.

E-mail address: munteanuaalexandru@gmail.com (A.M. Munteanu).

<http://dx.doi.org/10.1016/j.beproc.2016.03.009>

0376-6357/© 2016 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

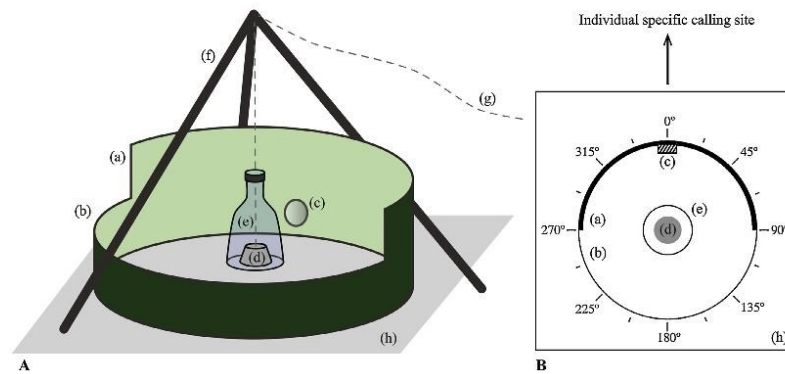


Fig. 1. Schematic representation of unequal-height arena with escape tunnel (condition three). A. Lateral view. B. Aerial view. The arena is composed of: (a) high wall, (b) low wall and (c) escape tunnel. The release device is composed of: (d) opaque cup, (e) transparent bottle, (f) modified tripod and (g) string. The arena floor (h) has angles marked in 22.5° increments. The arena was always positioned with starting angle (0°) pointing towards the focal individual's specific calling site.

Males defend long-term territories that they use as feeding grounds and mating sites (Narins et al., 2003; Ringler et al., 2012, 2009; Roithmair, 1992). Males produce advertisement calls from fixed calling sites (i.e. elevated structures above the forest floor) within their territories (Ringler et al., 2009). Like many dendrobatid frogs, male *A. femoralis* shuttle tadpoles from their territories to widely distributed aquatic deposition sites (Ringler et al., 2013). Experimentally translocated males return to their territories from up to 400 m in just a few days (Pašukonis et al., 2013) following a direct path (Pašukonis et al., 2014a, 2014b).

We conducted a field experiment to examine the way-finding flexibility of male *A. femoralis* in a detour task. More specifically, we analysed the escape routes of territorial males translocated from their calling sites to an enclosed circular arena. We hypothesized that translocated males would: 1) escape by jumping over the arena wall towards its former calling site, given an equal-height arena, 2) take a detour by jumping over the lower part of the wall, if a higher wall was facing the calling site and 3) prefer a straightforward path homeward, if available.

2. Material and methods

2.1. Study animals and location

We studied individual frogs from an *A. femoralis* population near the 'Saut Pararé' field camp (4°02'N, 52°41'W, WGS84) of the Nouragues Ecological Research Station (www.nouragues.cnrs.fr), in the nature reserve 'Les Nouragues', French Guiana. We carried out the study between 4th and 14th of February 2013, and the trials were performed between 10:00–18:30. This period marked the beginning of *A. femoralis* mating season when males were calling throughout the day and were motivated to return to their former calling site after displacement.

We captured 35 calling males, which were identified as territorial if they showed positive phonotaxis to a playback of a simulated male intruder. To avoid retesting the same frog, we identified frogs by their individual ventral patterns and noted the location of its calling site on a digital map (Ringler et al., 2014) using pocket computers (MobileMapper™ 10, Spectra-Precision, Westminster, Co., USA) with the ArcPad™ 10.0 (ESRI, Redlands, CA, USA) geographic information system. Promptly after capture we translocated the frogs a mean $\pm$ SE distance of 7.4 ± 0.78 m (range: 2–20 m) away from their calling sites, to a portable experimental arena, placed

near that male's home range. Each individual was naive in respect to the arena setup and was only tested once.

2.2. Experimental setup and procedure

The mobile circular arena ($\varnothing = 100$ cm) was made out of dark green plastic walls and a black plastic base. We marked the angles of the arena clockwise, in 22.5° incremental steps, on the plastic floor surface, outside of the arena. On location, we fixed the arena with two metal poles attached to the outer perimeter of the walls, and always oriented the 0° angle towards the translocated subject's original calling site (Fig. 1B).

We placed the subject in the centre of the arena under a releasing device consisting of an inner opaque wooden cup ($\varnothing = 6$ cm) and an outer transparent bottle ($\varnothing = 8$ cm) that could be lifted using a string run through a modified tripod above the arena (Fig. 1A). We kept each frog for two minutes under the opaque cup to habituate. We then allowed the frog a five-minute "orientation phase" under the transparent bottle with the opaque cup lifted, to avoid random escape behaviour. Subsequently, we lifted the entire releasing device and allowed the individual a "locomotory phase" of maximum 120 min to escape the arena. We counted a trial as completed once the frog touched the ground outside the arena. If the frog spent more than 120 min within the arena without escaping, we halted the trial and released the frog; such trials were coded as incomplete. Once outside the arena, we attempted to follow the frogs' movement direction, keeping a distance of approximately 3 m to avoid disturbing the individual. However, frogs were sometimes lost out of sight after a short distance due to the difficult observation conditions (small dark frog in low light forest understory) or when frogs hid behind forest floor structures (e.g., leaves, logs, roots).

We video-recorded the trials using a tripod-fixed camera filming from an elevated angle. Two experimenters, that were positioned 3 m away from the arena and in opposing angles, noted additional information on the frequency of movements, escape angle and strategy. We applied three consecutive test conditions to different frogs, respectively. In an equal-height arena test we assessed the choice of the escape route in relation to the calling site direction, by providing equally difficult escape possibilities from the arena at all angles, i.e. 20 cm high uniform walls ($n = 11$). We chose the wall height to be high enough that the frogs would not see their calling sites directly but low enough that the frogs would be able to jump over it. In an unequal-height obstacle arena test, we confronted 10 further individuals with a higher wall facing their calling site.

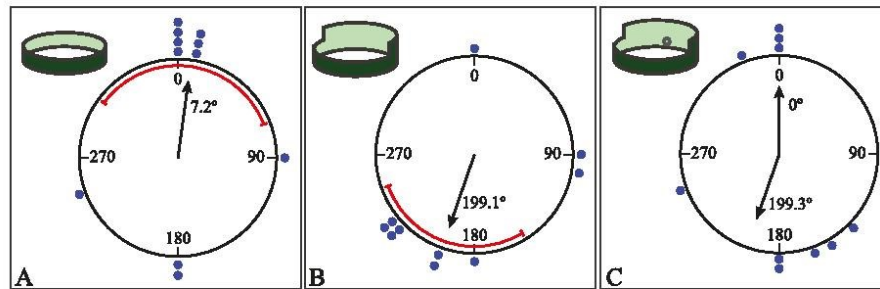


Fig. 2. Results of the arena experiments. A. Equal-height arena test. B. Unequal-height arena test. C. Unequal-height arena test with escape tunnel. Outer points represent escaped individuals and are placed at the corresponding escape angle; arrows represent the mean escape angle; and arc segments represent the 95% confidence interval. In the last experiment, the two arrows indicate the escape angle for three tunnel escapees (0°) and the mean escape angle for the over-the-wall escapees (199.3°).

For this purpose, we raised this semi-circular section of the wall to a height of 36 cm, while leaving the other half at 20 cm (Fig. 1A). During pilot trials we determined that frogs would not be able to jump over a wall of 36 cm but they could climb over it if no other escape possibility was available. We always rotated the centre of the high wall to 0° towards the initial calling location of the tested individual. Finally, in a third test we provided an additional escape route and tested 10 further individuals. For this, we fitted an arena identical to the unequal-height condition with a grey plastic tube ($\varnothing = 7$ cm) located at the centreline of the high wall, elevated 5 cm from the ground. Oriented at 0° , the tube provided a straightforward but narrow (snout-vent length of these frogs is approximately 3 cm) shortcut towards the focal individual's specific calling site.

2.3. Data analysis

Of 35 initiated trials, we excluded four trials from our analysis: three from the first test (in one case because heavy rain flooded the arena, in another case a branch, fallen during the experiment, provided an additional escape route, in one case the frog escaped underneath the arena wall), and one incomplete trial from the second test, when the subject did not manage to escape within the allocated maximum time.

This left 31 valid escape trials, which were coded from the video footage. During the locomotion phase we scored all movements, such as: hops, jumps, pivotal body orientations and wall climb attempts. For each frog, we summed the number of such movements, and also calculated the total time spent in the arena.

For statistical analysis, we measured the variation in the angles about the mean direction with a V-test for circular uniformity (with expected mean directions of 0° in the equal-height arena and of 180° in the unequal-height arena) in the circular statistics software Oriana v. 4.02 (Kovach Computing Services, Pentraeth, Wales, UK). In addition, we used the Oriana software to calculate the correlation coefficient between the escape angle and both movement and time spent in the arena using the circular-linear correlation.

Because of the wide spread of the mean vectors, we further tested for homeward escape in the standard arena with a Chi-squared test on quadrant grouped escape angles. All linear statistics were done with SPSS 20.0.

3. Results

Frogs escaped the arena either by climbing up the wall, or by jumping onto the edge of the wall and then outside, or by a combination of jumping and climbing. We found no significant difference in time spent in the arena (Kruskal-Wallis test: $H = 3.45$, d.f. = 2,

$p = 0.178$) or number of movements (Kruskal-Wallis test: $H = 1.58$, d.f. = 2, $p = 0.454$) across the three experimental conditions. We also found no correlation between time spent in the arena and the escape angle (Spearman's $\rho = 0.099$, $p = 0.603$), or movement frequency and escape angles (Spearman's $\rho = -0.35$, $p = 0.854$). However, time spent in the arena and number of movements was correlated (Spearman's $\rho = 0.654$, $p < 0.000$); unsurprisingly, frogs that took longer to escape also made more movements in the arena.

3.1. Equal-height arena test

When faced with a uniform height of the arena wall, the frogs predominantly chose an escape path towards the calling site (Mean = 7.2° , 95% CI = 306.5° – 67.9° , V-Test $p = 0.024$, $n = 11$). Out of the 11 individuals, 7 escaped via the 90° -quadrant facing towards the calling site, which differs significantly from a uniform random choice ($\chi^2 = 9$, d.f. = 3, $p = 0.029$, Fig. 2A). Thus, all things being equal, frogs chose to escape in the direction of their former calling site.

3.2. Unequal-height obstacle arena test

However, when confronted with a high wall facing their initial calling site, the majority of the frogs ($n = 9$ out of 10) avoided the higher obstacle and instead escaped via the lower wall. One individual climbed over the high wall (Mean = 199.1° , 95% CI = 148.7° – 249.5° , V-Test $p = 0.016$, $n = 10$) ($\chi^2 = 6.4$, $p = 0.011$, Fig. 2B), showing that escape over the higher wall was physically possible. Once outside the arena, out of 9 frogs that escaped over the low wall, 7 readjusted their trajectory towards their former calling site, while the other 2 individuals moved away in different directions.

3.3. Unequal-height obstacle arena test with escape tunnel

In the arena test with an additional escape tunnel, frogs predominantly climbed over the low wall ($n = 6$), while only three frogs used the escape tunnel ($\chi^2 = 3.8$, $p = 0.150$, Fig. 2C). Again one individual climbed the high wall facing homeward. Outside the arena, out of 6 frogs that escaped over the low wall, 4 readjusted their trajectory towards their former calling site, while 2 went in different directions.

4. Discussion

We found that male *A. femoralis* preferred to escape towards their former calling site when placed in an arena with uniform wall height. However, when confronted with a higher wall facing

the calling site, frogs mostly chose to detour by jumping over the lower wall. They continued to prefer this “detour escape” even in the presence of an additional shortcut passage facing directly towards their initial calling site.

As expected, the former calling site seems to be a powerful motivator for *A. femoralis* and a reliable objective in a detour task comparable to food for jumping spiders (Tarsitano and Jackson, 1994) or green toads (Collett, 1982), and social rewards for chicken (Regolin et al., 1995). Frogs appear to be capable of avoiding potentially energetically expensive obstacles. However, this task was not physically inevitable: the higher obstacle was not impossible to overcome, as shown by two individuals who chose to escape over the higher wall towards their former calling site, further supporting the results from the arena with equally high walls. Also, almost half of the tested frogs immediately went towards their former calling sites, touched the wall repeatedly, and looked up before deciding where to escape. Interestingly, the majority of the tested males seemed to “evaluate” the situation. They stayed in the centre of the arena while turning or hopping in different directions, and then escaped over the low wall on the first try, suggesting a strategic avoidance of the higher obstacle. In hindsight, an equal-height arena with high walls (i.e. 36 cm) would have been a valuable additional control to assess frogs’ ability and motivation to choose the homeward direct escape route, without an alternative route available. However, with limited time in the field and limited knowledge of *A. femoralis* jumping capabilities, we chose the low wall as a safer and still informative control condition.

Contrary to our expectations, the majority of individuals from the third test did not choose the tunnel as an escape route. In an earlier study, green toads (*P. viridis*) predominantly used the shortcut through an obstacle, but this was represented by a simple gap in a palisade (Collett, 1982). This difference might be due to the fact that frogs in our arena did not have a direct sight of the calling perch through the elevated tunnel. Without the direct sight outside, the confined tunnel might have not appeared as a valid or safe escape route to the frogs.

Our results on homeward escape orientation are in line with a previous study that demonstrated the striking ability of males of this species to take a direct trajectory to return to their territory after being translocated up to 360 m (Pašukonis et al., 2014b). However, in another study the authors did not find an initial homeward orientation in a similar arena setup when relocating frogs over a short distance (<35 m) from their territory (Pašukonis et al., 2014a). In our study we found that translocating *A. femoralis* males over a mean distance of seven metres from their calling site leads to the majority of frogs choosing to escape towards their former calling site. We additionally observed that most individuals that did not escape directly towards home, readjusted their movement once outside the arena, and then headed towards their former calling site. A few individuals did not immediately home back, but hid in the leaf litter after their escape. These individuals might not have been motivated to call again after the trial and thus did not show immediate orientation towards the calling site. Given that *A. femoralis* males of the study population occupy territories averaging 14 m in diameter, and territory size varies largely between individuals (Ringle et al., 2011; Roithmair, 1992) it is likely that, in many cases, our arena was set up within the focal male’s defended and safe area. In general, the tested subjects were seen calling in their territory during the next days.

Previously, amphibian detour behaviour has been documented only under laboratory conditions (Collett, 1982; Lock and Collett, 1979). Our study represents an attempt at an experimental demonstration of anuran detour behaviour in the field. We found that *A. femoralis* males are capable of avoiding obstacles in a detour task in their natural habitat towards reaching an ecologically relevant goal—their calling site. Poison frog ecology involves territory

defense, tadpole transport and calling site selection in a dynamic environment, the rainforest, and consequently demands a degree of way-finding flexibility. Together with several recent studies on *A. femoralis* (Pašukonis et al., 2014b; Ringle et al., 2015) our results address the incomplete picture of spatial cognitive flexibility in amphibians. Our study introduces the use of translocation and portable arenas as a simple new method suitable for studying amphibian behavioural plasticity in the field—a topic that merits further investigation.

Acknowledgements

The authors would like to thank Julia Felling for her help in the field, Max Ringle for valuable comments on the experimental setup and the manuscript, the Pararé staff for logistical support, and two anonymous reviewers for their helpful comments on a previous version of the manuscript. Our study was supported by the Austrian Science Fund (FWF): project W1234, P25612 and P24788-B22. The Nouragues Ecological Research Station is supported by CNRS. Our study was approved by the scientific committee of the Nouragues Ecological Research Station and all necessary permits for the study were granted by the Centre National de la Recherche Scientifique (CNRS) and by the Direction Regionale de l’Environnement de Guyane (DIREN).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.beproc.2016.03.009>.

References

- Amézquita, A., Lima, A.P., Jehle, R., Castellanos, L., Ramos, Ó., Crawford, A.J., Gasser, H., Hödl, W., 2009. Calls, colours, shape, and genes: a multi-trait approach to the study of geographic variation in the Amazonian frog *Allobates femoralis*. *Biol. J. Linn. Soc.* 98, 826–838. <http://dx.doi.org/10.1111/j.1095-8312.2009.01324.x>.
- Baragli, P., Vitale, V., Paoletti, E., Sighieri, C., Reddon, A.R., 2010. Detour behaviour in horses (*Equus caballus*). *J. Ethol.* 29, 227–234. <http://dx.doi.org/10.1007/s10164-010-0246-9>.
- Bisazza, A., Pignatti, R., Vallortigara, G., 1997. Laterality in detour behaviour: interspecific variation in poeciliid fish. *Anim. Behav.* 54, 1273–1281.
- Chapuis, N., Varlet, C., 1987. Short cuts by dogs in natural surroundings. *Q. J. Exp. Psychol. Sect. B* 39, 49–64. <http://dx.doi.org/10.1080/14640748708402251>.
- Chapuis, N., Thinus-Blanc, C., Poucet, B., 1983. Dissociation of mechanisms involved in dogs oriented displacements. *Q. J. Exp. Psychol. Sect. B* 35 (Pt 3), 213–219.
- Collett, T.S., 1982. Do toads plan routes? A study of the detour behaviour of *Bufo viridis*. *J. Comp. Physiol.* 146, 261–271. <http://dx.doi.org/10.1007/BF00610246>.
- Collett, T.S., 1996. Insect navigation en route to the goal: multiple strategies for the use of landmarks. *J. Exp. Biol.* 199, 227–235.
- Ingle, D., 1970. Visuomotor functions of the frog optic tectum. *Brain, Behav. Evol.* 3, 57–71.
- Ingle, D., 1973. Two visual systems in the frog. *Science* 181, 1053–1055. <http://dx.doi.org/10.1126/science.181.4104.1053>.
- Köhler, W., 1999. The Mentality of Apes. Psychological Bulletin. Routledge, Trench, Trubner & Co., London. <http://dx.doi.org/10.1037/h0064067>.
- Kimchi, T., Terkel, J., 2003. Detours by the blind mole-rat follow assessment of location and physical properties of underground obstacles. *Anim. Behav.* 66, 885–891. <http://dx.doi.org/10.1006/anbe.2003.2267>.
- Kohlsdorf, T., Biewener, A.A., 2006. Negotiating obstacles: running kinematics of the lizard *Sceloporus malachiticus*. *J. Zool.* 270, 359–371. <http://dx.doi.org/10.1111/j.1469-7998.2006.00150.x>.
- Lock, A., Collett, T., 1979. A toad’s devious approach to its prey: a study of some complex uses of depth vision. *J. Comp. Physiol.* 131, 179–189. <http://dx.doi.org/10.1007/BF00619078>.
- Lock, A., Collett, T., 1980. The Three-Dimensional world of a toad. *Proc. R. Soc. B Biol. Sci.* 206, 481–487. <http://dx.doi.org/10.1098/rspb.1980.0009>.
- Lustig, A., Ketter-Katz, H., Katzir, G., 2013. Lateralization of visually guided detour behaviour in the common chameleon *Chamaeleo chamaeleon*, a reptile with highly independent eye movements. *Behav. Process.* 100, 110–115. <http://dx.doi.org/10.1016/j.beproc.2013.08.002>.
- Narins, P.M.P., Hödl, W., Grabul, D.S.D., 2003. Bimodal signal requisite for agonistic behavior in a dart-poison frog *Epipedobates femoralis*. *Proc. Natl. Acad. Sci. U. S. A.* 100, 577–580. <http://dx.doi.org/10.1073/pnas.0237165100>.

- Nesterova, A., Hansen, F., 2009. Simple and integrated detours: field tests with Columbian ground squirrels. *Anim. Cogn.* 12, 655–670, <http://dx.doi.org/10.1007/s10071-009-0224-1>.
- Pašukonis, A., Ringler, M., Brandl, H.B., Mangione, R., Ringler, E., Hödl, W., 2013. The homing frog: high homing performance in a territorial dendrobatid frog *Allobates femoralis* (Dendrobatidae). *Ethology* 119, 762–768, <http://dx.doi.org/10.1111/eth.12116>.
- Pašukonis, A., Loretto, M.-C., Landler, L., Ringler, M., Hödl, W., 2014a. Homing trajectories and initial orientation in a Neotropical territorial frog *Allobates femoralis* (Dendrobatidae). *Front. Zool.* 11, 29, <http://dx.doi.org/10.1186/1742-9994-11-29>.
- Pašukonis, A., Warrington, I., Ringler, M., Hödl, W., 2014b. Poison frogs rely on experience to find the way home in the rainforest. *Biol. Lett.*, 10.
- Poucet, B., Thinus-Blanc, C., Chapuis, N., 1983. Route planning in cats: in relation to the visibility of the goal. *Anim. Behav.* 31, 594–599.
- Regolin, L., Vallortigara, G., Zanforlin, M., 1994. Perceptual and motivational aspects of detour behaviour in young chicks. *Anim. Behav.* 47, 123–131, <http://dx.doi.org/10.1006/anbe.1994.1014>.
- Regolin, L., Vallortigara, G., Zanforlin, M., 1995. Detour behaviour in the domestic chick: searching for a disappearing prey or a disappearing social partner. *Anim. Behav.* 50, 203–211, <http://dx.doi.org/10.1006/anbe.1995.0232>.
- Ringler, M., Ursprung, E., Hödl, W., 2009. Site fidelity and patterns of short- and long-term movement in the brilliant-thighed poison frog *Allobates femoralis* (Aromobatidae). *Behav. Ecol. Sociobiol.* 63, 1281–1293, <http://dx.doi.org/10.1007/s00265-009-0793-7>.
- Ringler, M., Ringler, E., Magaña Mendoza, D., Hödl, W., 2011. Intrusion experiments to measure territory size: development of the method, tests through simulations, and application in the frog *Allobates femoralis*. *PLoS One* 6, e25844, <http://dx.doi.org/10.1371/journal.pone.0025844>.
- Ringler, E., Ringler, M., Jehle, R., Hödl, W., 2012. The female perspective of mating in *A. femoralis*, a territorial frog with paternal care—a spatial and genetic analysis. *PLoS One* 7, e40237, <http://dx.doi.org/10.1371/journal.pone.0040237>.
- Ringler, E., Pašukonis, A., Hödl, W., Ringler, M., 2013. Tadpole transport logistics in a Neotropical poison frog: indications for strategic planning and adaptive plasticity in anuran parental care. *Front. Zool.* 10, 67, <http://dx.doi.org/10.1186/1742-9994-10-67>.
- Ringler, M., Mangione, R., Pašukonis, A., Rainer, G., Gyimesi, K., Felling, J., Kronaus, H., Réjou-Méchain, M., Chave, J., Reiter, K., Ringler, E., 2014. High-resolution forest mapping for behavioural studies in the Nature Reserve Les Nouragues, French Guiana. *J. Maps*, 1–7, <http://dx.doi.org/10.1080/17445647.2014.972995>.
- Ringler, E., Pašukonis, A., Fitch, W.T., Huber, L., Hödl, W., Ringler, M., 2015. Flexible compensation of uniparental care: female poison frogs take over when males disappear. *Behav. Ecol.* 26, 1219–1225, <http://dx.doi.org/10.1093/beheco/arv069>.
- Roithmair, M.E., 1992. Territoriality and male mating success in the dart-poison frog, *Epipedobates femoralis* (Dendrobatidae, Anura). *Ethology* 92, 331–343, <http://dx.doi.org/10.1111/j.1439-0310.1992.tb00970.x>.
- Sinsch, U., 2014. Movement ecology of amphibians: from individual migratory behaviour to spatially structured populations in heterogeneous landscapes. *Can. J. Zool.* 92, 491–502, <http://dx.doi.org/10.1139/cjz-2013-0028>.
- Smith, B.P., Litchfield, C.A., 2010. How well do dingoes *Canis dingo*, perform on the detour task? *Anim. Behav.* 80, 155–162, <http://dx.doi.org/10.1016/j.anbehav.2010.04.017>.
- Tarsitano, M., Jackson, R., 1994. Jumping spiders make predatory detours requiring movement away from prey. *Behaviour* 131, 65–73.
- Tarsitano, M.S., Jackson, R.R., 1997. Araneophagic jumping spiders discriminate between detour routes that do and do not lead to prey. *Anim. Behav.* 53, 257–266, <http://dx.doi.org/10.1006/anbe.1996.0372>.
- Tarsitano, M.S., 2006. Route selection by a jumping spider (*Portia labiata*) during the locomotory phase of a detour. *Anim. Behav.* 72, 1437–1442, <http://dx.doi.org/10.1016/j.anbehav.2006.05.007>.
- Wynne, C.D.L., Leguet, B., 2004. Detour behavior in the Quokka (*Setonix brachyurus*). *Behav. Process.* 67, 281–286, <http://dx.doi.org/10.1016/j.beproc.2004.04.007>.
- Zucca, P., Antonelli, F., Vallortigara, G., 2005. Detour behaviour in three species of birds: quails (*Coturnix* sp.), herring gulls (*Larus cachinnans*) and canaries (*Serinus canaria*). *Anim. Cogn.* 8, 122–128, <http://dx.doi.org/10.1007/s10071-004-0243-x>.



Identification, synthesis and mass spectrometry of a macrolide from the African reed frog *Hyperolius cinnamomeoventris*

Markus Menke^{1,§}, Pardha Saradhi Peram^{1,§}, Iris Starnberger², Walter Hödl², Gregory F.M. Jongsma³, David C. Blackburn³, Mark-Oliver Rödel⁴, Miguel Vences⁵ and Stefan Schulz^{*1}

Full Research Paper

[Open Access](#)

Address:

¹Technische Universität Braunschweig, Institute of Organic Chemistry, Hagenring 30, 38106 Braunschweig, Germany, ²Department for Integrative Zoology, Althanstraße 14, 1090 Vienna, Austria, ³Florida Museum of Natural History, University of Florida, Gainesville, Florida 32611, United States of America, ⁴Museum für Naturkunde, Leibniz Institute for Evolution and Biodiversity Science, Invalidenstr. 43, 10115 Berlin, Germany and ⁵Technische Universität Braunschweig, Institute of Zoology, 38106 Braunschweig, Germany

Email:

Stefan Schulz* - stefan.schulz@tu-bs.de

* Corresponding author

§ Both authors share first authorship.

Keywords:

chemical communication; chiral gas chromatography; macrocyclic lactones; ring-closing metathesis; pheromones

Beilstein J. Org. Chem. 2016, 12, 2731–2738.

doi:10.3762/bjoc.12.269

Received: 28 September 2016

Accepted: 02 December 2016

Published: 13 December 2016

This article is part of the Thematic Series "Lipids: fatty acids and derivatives, polyketides and isoprenoids".

Guest Editor: J. S. Dickschat

© 2016 Menke et al.; licensee Beilstein-Institut.

License and terms: see end of document.

Abstract

The contents of the gular glands of the male African reed frog *Hyperolius cinnamomeoventris* consist of a mixture of aliphatic macrolides and sesquiterpenes. While the known macrolide gephyromantolide A was readily identified, the structure of another major component was suggested to be a tetradecen-13-olide. The synthesis of the two candidate compounds (*Z*)-5- and (*Z*)-9-tetradecen-13-olide revealed the former to be the naturally occurring compound. The synthesis used ring-closing metathesis as key step. While the Hoveyda–Grubbs catalyst furnished a broad range of isomeric products, the (*Z*)-selective Grubbs catalyst lead to pure (*Z*)-products. Analysis by chiral GC revealed the natural frog compound to be (5*Z*,13*S*)-5-tetradecen-13-olide (**1**). This compound is also present in the secretion of other hyperoliid frogs as well as in femoral glands of male mantellid frogs such as *Spinomantis aglavei*. The mass spectra of the synthesized macrolides as well as their rearranged isomers obtained during ring-closing metathesis showed that it is possible to assign the location of the double bond in an unsaturated macrolide on the basis of its EI mass spectrum. The occurrence of characteristic ions can be explained by the fragmentation pathway proposed in the article. In contrast, the localization of a double bond in many aliphatic open-chain compounds like alkenes, alcohols or acetates, important structural classes of pheromones, is usually not possible from an EI mass spectrum. In the article, we present the synthesis and for the first time elucidate the structure of macrolides from the frog family Hyperoliidae.

Introduction

The lactone motif is found in many compounds that are used in chemical communication. Among them, macrocyclic lactones are an important class because of their biosynthetic availability and their inherent compound properties. During the biosynthesis of macrocyclic lactones, a fatty acid precursor is often oxidized near the end of the chain to form a polar hydroxy acid. The following ring-closure reduces the hydrophilicity of the compound and increases its vapor pressure, making the resulting macrocycle well-suited to serve as a signal [1]. Fatty acid derived macrolactones were therefore repeatedly invented during evolution and are used by different animals such as bees, beetles, butterflies, cockroaches, or frogs as pheromones [1].

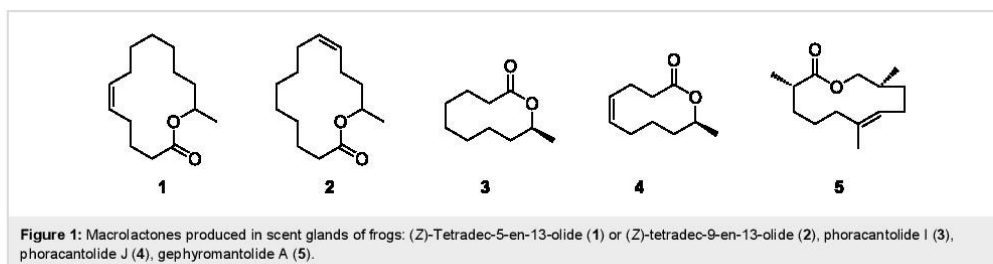
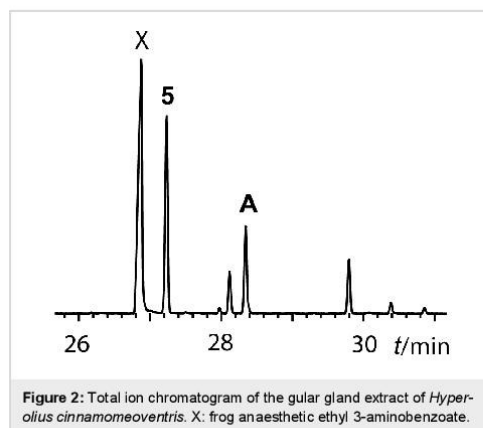
Finding and choosing mates in frogs is usually regarded as being primarily acoustically mediated. Nevertheless, some families like the Mantellidae from Madagascar also use chemical cues. Macrocyclic lactones such as phoracantholide I (3), phoracantholide J (4) or gephyromantolide A (5), are released by males from femoral glands to serve as signals, often accompanied by secondary alcohols (Figure 1) [2–4]. Another frog family most likely using volatile compounds during courtship are the African reed frogs, Hyperoliidae. The males of most species emit acoustic cues to attract females. During the call, an often conspicuously colored gland on their vocal sac (the gular gland), only innervated during the mating season in at least some species, releases a complex blend of volatiles when exposed [5]. A first analysis revealed the presence of mostly unknown terpenes, macrolides and other components in species specific compositions [5].

Detailed exploration of the function of these frog volatiles requires the analysis of the secretion and the synthesis of the identified compounds for biological testing. Because of the small amount of natural material available, only GC–MS investigations can be used to identify the compounds. The analysis revealed unsaturated macrocyclic lactones to be major constituents of the secretion of the cinnamon-bellied reed frog, *Hyperolius cinnamomeoventris*. The location and configuration of the double bond might be relevant for their function, as it is

the case in many insect pheromones. In typical pheromone components of insects, including long chain alkenes, alkenols, or unsaturated aldehydes, the location of the double bond can usually not be determined by analysis of their mass spectra. Positional isomers often exhibit almost identical spectra [6] and derivatization or special mass spectrometric techniques are needed to localize double bonds [7–9]. Because this might also be the case for macrolides [10], it is of interest for their identification to compare mass spectra of positional isomers of unsaturated macrolactones. Furthermore, a fast synthetic strategy is needed to synthesize various isomers. In the present work we describe 1) the identification of macrolides from *H. cinnamomeoventris*, 2) discuss EI mass spectra of unsaturated macrolactones, and 3) show how ring-closing metathesis reaction conditions can be selected to obtain either pure compounds or a library of compounds useful for evaluation of their mass spectra.

Results and Discussion

The GC–MS analysis of a gular gland extract of *Hyperolius cinnamomeoventris* showed the presence of several unknown compounds, sesquiterpenes and macrolides (Figure 2).



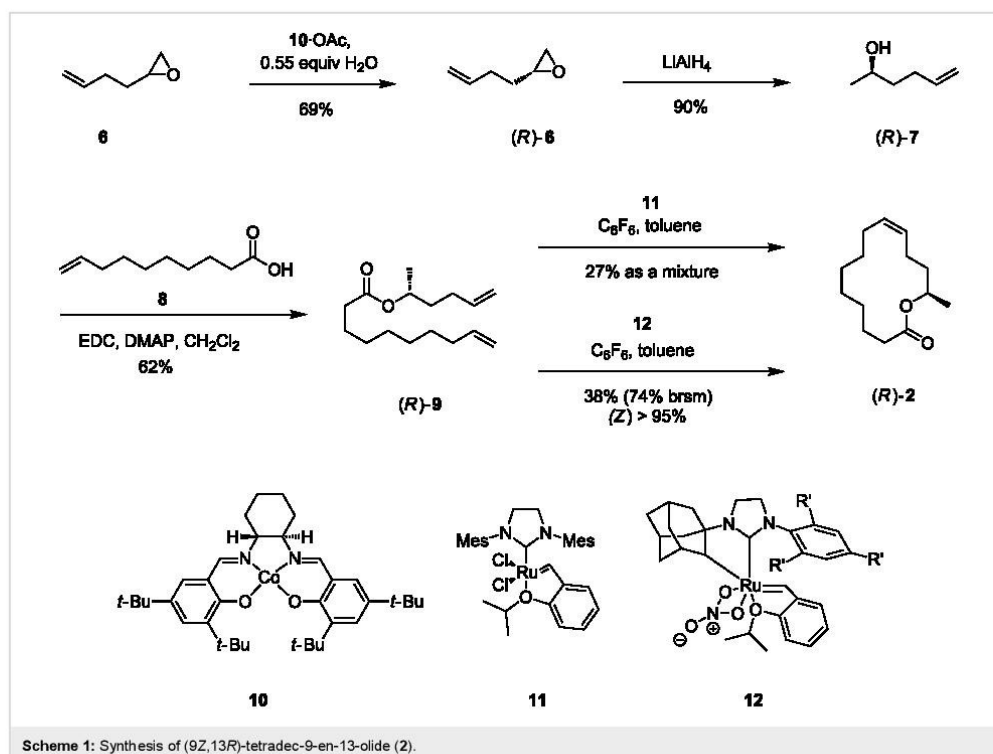
Gephyromantolide A (**5**), known from the mantellid frog *Gephyromantis boulengeri* [4], was readily identified. A second macrolide (**A** in Figure 2) showed a mass spectrum similar to that reported for (*Z*)-tetradec-5-en-13-olide (**1**) by Millar et al. [11], exhibiting a molecular ion at m/z 224. This macrolide, called cucujolide III, is used by the flat grain beetle *Cryptolestes pusillus* as pure (*S*)-enantiomer and by *C. turcicus* as a 33:67 *R/S* mixture [12], and acts as a synergist to the respective pheromones [11,13]. Microhydrogenation of the frog extract furnished 13-tetradecanolide, thus confirming compound **A** to be a 13-tetradecenolide. The double bond position in this macrolide likely is at C-5, because of the similarity of the mass spectrum to that of the beetle macrolide. Nevertheless, because locating double bonds in such compounds based on the mass spectrum alone seemed not to be reliable, we opted to synthesize two positional isomers.

Compound **1** is biosynthetically formed by the *Cryptolestes* beetles starting from oleic acid [14] that is shortened to 5-tetradecenoic acid, followed by $\omega-1$ oxidation and ring closure [15]. Another possibility would be that a common saturated acid such as stearic acid is chain-shortened first to tetradecanoic acid, on which a common Δ^9 -desaturase is acting,

leading after $\omega-1$ oxidation and ring closure to tetradec-9-en-13-olide (**2**). Therefore, we opted to synthesize **2** as well.

To allow later enantiomer determination of **A**, an enantioselective synthetic strategy was followed. Several synthetic routes for the synthesis of **1** have been reported [16–20]. These syntheses were performed before the advent of ring-closing metathesis (RCM), requiring more than 10 steps each. RCM can shorten the synthesis remarkably, but requires careful selection of the RCM catalyst to control the double bond configuration. For example, Fürstner and Langemann obtained *rac*-**1** in 31:69 (*E/Z*)-mixture using a Ru-carbene type catalyst similar to a Grubbs I catalyst [21].

The synthesis of (*R*)-**2** using RCM as key step is shown in Scheme 1. Enantiomerically pure 1,2-epoxyhex-5-ene (**6**) was obtained by Jacobsen hydrolytic kinetic resolution on commercially available **6** (Scheme 1) [22,23]. Surprisingly, the yield of 69% of the (*R*)-enantiomer was higher than the theoretically upper limit of 50%. We discovered that **6**, sold by Acros Organics as racemic compound, was actually enriched in the



desired (*R*)-enantiomer (see Supporting Information File 1 for optical rotation values). Diene (*R*)-9 was obtained by reduction of the epoxide **6** with LiAlH_4 to form alcohol (*R*)-7, followed by esterification with 9-decenoic acid (**8**) using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 4-(dimethylamino)pyridine (DMAP) [24,25]. The following RCM was performed using Grubbs–Hoveyda II catalyst (**11**) and hexafluorobenzene as an additive [26]. During the reaction isomerization occurred, leading to a mixture of positional isomers and chain shortened as well as elongated products. Addition of *p*-benzoquinone [27] and improved purification methods [28] suppressed isomer formation only partially. Replacing catalyst **11** with the Grubbs second generation catalyst (1,3-bis(2,4,6-trimethylphenyl)-2-imidazolidinylidene)dichloro(phenylmethylene)(tricyclohexylphosphine)ruthenium reduced the isomerization, leading to an (*E/Z*)-mixture of **2**. Finally, the (*Z*)-selective Grubbs catalyst **12** furnished the best results [29,30]. This catalyst yielded only the desired product (*R*)-**2** with a (*Z*)-configured double bond, although in moderate yield (see Supporting Information File 1 for full experimental data).

A similar synthetic strategy was applied for the synthesis of macrolide **1** (Scheme 2). The stereogenic center was introduced using commercially available (*R*)-propylene oxide (**13**) as starting material. After copper-catalyzed opening of the epoxide with 6-heptenylmagnesium bromide obtained from 7-bromo-1-heptene (**14**) and Steglich esterification with 5-hexenoic acid (**16**), RCM using (*Z*)-selective Grubbs catalyst **12** was used to synthesize macrolide (*R*)-**1** without any isomerization. Comparison of the mass spectra (Figure 3) and gas chromatographic retention times of pure (*Z*)-**1**, the (*E/Z*)-mixture obtained by the Hoveyda–Grubbs II catalyst, and **2** with those of the natural compound proved the frog compound to be (*Z*)-tetradec-5-en-13-olide (**1**).

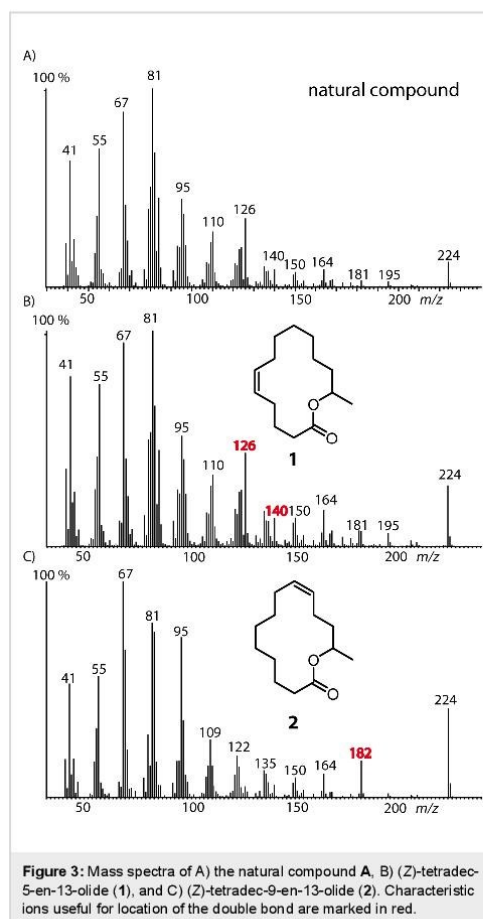
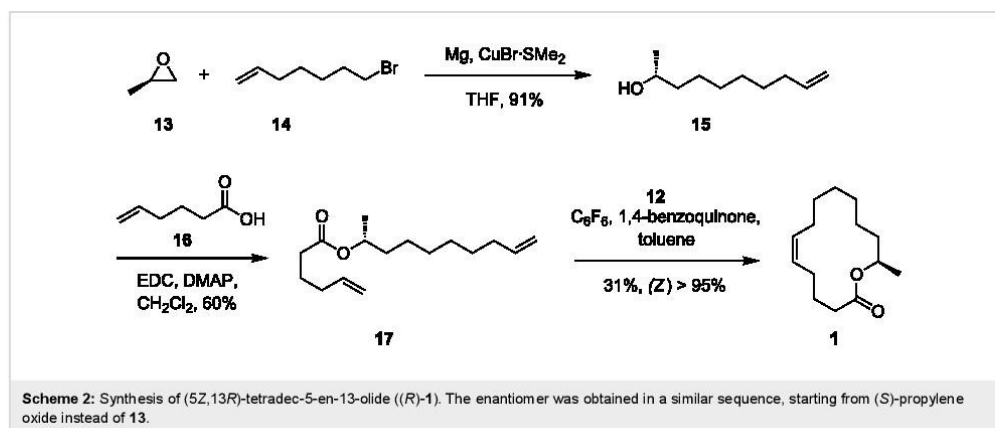


Figure 3: Mass spectra of A) the natural compound A, B) (*Z*)-tetradec-5-en-13-olide (**1**), and C) (*Z*)-tetradec-9-en-13-olide (**2**). Characteristic ions useful for location of the double bond are marked in red.



Scheme 2: Synthesis of (*5Z,13R*)-tetradec-5-en-13-olide ((*R*)-**1**). The enantiomer was obtained in a similar sequence, starting from (*S*)-propylene oxide instead of **13**.

For the determination of the absolute configuration of the natural compound, the other enantiomer (*S*)-**1** was needed as well. It was synthesized according to the synthesis shown in Scheme 2, starting from (*S*)-propylene oxide instead of the (*R*)-enantiomer **13**. The stereochemistry was determined by chiral gas chromatography as shown in Figure 4. The coinjection of pure (*S*)-**1** with the racemic mixture proved the first eluting peak to be this enantiomer. Injection of the natural sample as well as (*S*)-**1** showed both compounds to be enantiomerically pure. Finally, coinjection of both samples showed their identity.

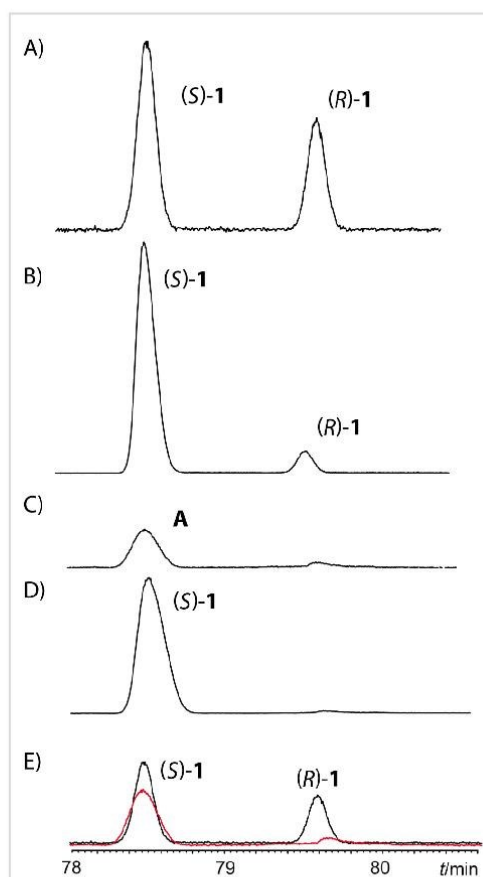


Figure 4: Total ion chromatogram of the enantiomer separation of (*Z*)-**1** on a chiral β -TBDMS-Hydrodex phase. Temperature program: isothermal for 60 min at 110 °C, then with 2 °C/min to 160 °C, followed by a sharp ramp with 25 °C/min to 220 °C. A) Racemic mixture, B) coinjection of racemic mixture and (*S*)-**1**, C) natural extract, D) coinjection of the natural extract and (*S*)-**1**, E) Overlay of A and C showing that the minor peak present in the extract is not the (*R*)-enantiomer of **1**. Black: racemate, red: natural extract. Peak identities were confirmed by mass spectrometry.

With the synthetic material in hand, we analyzed the EI mass spectra of **1** and **2**. No significant differences were detectable between the *E/Z*-isomers of each compound. Nevertheless, contrary to open chain compounds, characteristic differences could be found for the positional isomers. While most ions are similar in both spectra, the prominent ion m/z 126 present in **1** is shifted to m/z 182 in **2**. These ions can be used to assign the location of the double bond in the macrocyclic ring.

A possible fragmentation pathway leading to these ions is shown in Figure 5. One can speculate that after ionization of the double bond in **1** an allylic cleavage occurs, leading to the radical cation **18** along pathway a. An additional bond cleavage of a C–O single bond releases a neutral molecule, e.g., methylcyclohexane, giving rise to ion m/z 126 (**20**). High-resolution mass spectral data support the hypothesis because the ions m/z 126 as well as m/z 182 (**25**) formed from **2** have a molecular composition of $C_7H_{10}O_2$ (HRMS: 126.07005 found, calcd. 126.0681) or $C_{11}H_{18}O_2$ (HRMS: found 182.13206, calcd. 182.1307), respectively. Ion **20** is accompanied by the ion m/z 140 (HRMS: found 140.08545, calcd. 140.08373) of the same ion series $C_nH_{2n-4}O_2$ that occurs in lower abundance. Its formation along pathway b can be explained by homoallylic cleavage of **18** into **21** and further fragmentation into **22**. The formation of the ions of this series is obviously determined by the position of the double bond in the chain. Therefore, ion m/z 182 of **2** arises by the same mechanism, indicating the location of the double bond at C-9 (Figure 5). A b-type ion cannot be formed, because pathway b is not operative due to the close proximity of the C–O group. The pathways shown in Figure 5 are speculative. Another possible mechanism leading to the same ions and starting with ionization of the C–O oxygen atom instead of the double bond is shown in Supporting Information File 1 (see Figure S1). Nevertheless, the proposal is useful to predict the position of double bonds in mono-unsaturated macrolides. This position can be deduced from the ion $C_{n+4}H_{2n+4}O_2$, generated by a double bond in the $n + 2$ position, as shown in **26**.

We subsequently tested our method with mass spectra of other macrolides previously reported or present in our compound library. In the mass spectra of (*Z*)-octadec-9-en-13-olide [31] and octadec-9-en-17-olide peaks at m/z 182 with higher and m/z 196 with lower intensity are present, consistent with a C-9 double bond. In contrast, (*Z*)-octadec-11-en-13-olide does not show such ions, because the close proximity of the double bond and the C–O group does not allow fragmentation along pathway a or b. The spectra of 4-methyl-5-decen-9-olide and 8-methyl-5-decen-9-olide [3] exhibit the expected ions m/z 140 and m/z 126, respectively. Pathway b is not operative because of the proximity of the C–O group. A slight alteration of the frag-

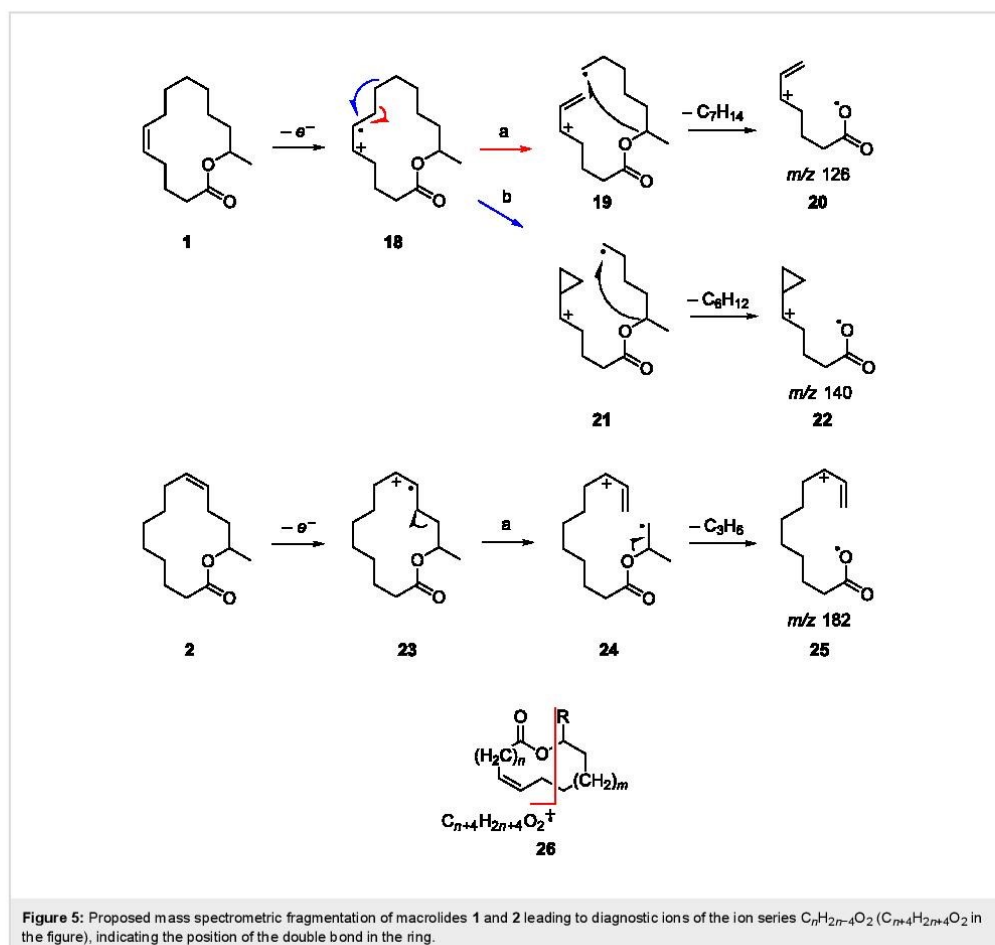


Figure 5: Proposed mass spectrometric fragmentation of macrolides 1 and 2 leading to diagnostic ions of the ion series $C_nH_{2n-4}O_2$ ($C_{n+4}H_{2n+4}O_2$ in the figure), indicating the position of the double bond in the ring.

mentation can be observed if the double bond is located at C-4, as in phoracantholide J, 4-decen-9-olide [32]. Now in pathway a, additionally an H-atom is transferred leading to the uneven ion $C_nH_{2n-3}O_2$, m/z 113, while pathway b remains unchanged, furnishing m/z 126. In the methylated analog 6-methyl-4-decen-9-olide [3], these ions shift to m/z 127 and 140. If the double bond moves closer to the C=O group as in 3-dodecen-11-olide [33], both ions a and b are visible, but their abundance is so low that their diagnostic value is largely decreased. Another limitation is that mass spectra of unsaturated unbranched macrolactones, formally derived from ω -hydroxy acids, do not show the characteristic ions generated by pathways a or b. Obviously, the primary C–O bond is not attacked by intermediates like 19. Nevertheless, the mass spectrometric interpretation presented here will largely ease the identification of a broad range of

unsaturated macrolides. The procedure may also be applicable for di- or triunsaturated compounds.

We then focused on the products of the RCM using the Hoveyda–Grubbs II catalyst 11. It is well known that during RCM isomerization of the double bond might occur, especially with the Grubbs second generation catalysts, leading to products with migrated double bonds or smaller ring size [27,34–36]. Both types of products, 11-dodecenolides, 12-tridecenolides and isomerized 13-tetradecenolides were obtained. In addition, ring expanded 14-pentadecenolides were observed. The latter can be explained by dimerization of esters 9 or 17, isomerization of the double bond, and final ring closure, leading to either ring-contracted or expanded products. Such processes have been reported before in polymerization experiments using Grubbs cata-

lysts [36–38]. While the isomerization is usually regarded as a negative side reaction in RCM, it turned out to be advantageous for the identification of naturally occurring macrolides in our hands. The isomerized mixtures constitute a library of closely related macrolides differing slightly in position of double bonds and ring size. With the diagnostic mass spectrometric ions discussed above, each compound can be assigned a structure after GC–MS analysis. These mass spectra together with the gas chromatographic retention index are stored in our EIMS database, easing identification of similar compounds in the future. Using this approach, we could collect mass spectra of 5-dodecen-11-olide, 5- and 6-tridecen-12-olide, 5- to 9-tetradecen-13-olide, as well as 5- and 6-pentadec-14-olide (see Supporting Information File 1 for some spectra). This set of mass spectra allowed the identification of macrolides in other frog species.

We also found macrolide **1** in the gular glands of other hyperoliids including *H. concolor*, *H. adametzi*, and *Afrrixalus dorsalis*. It occurs also outside this frog family. It is a major constituent of the femoral gland of the mantellid frog *Spinomantis aglavei*, again in (*S*)-configuration. Minor amounts of **1** were also present in the glands of an undescribed species of *Güibemantis* similar to *G. bicalcaratus* as well as in *Gephyromantis ceratophrys*, accompanied by the isomer 8-tetradecen-13-olide in the latter species.

Conclusion

RCM using the (*Z*)-selective Grubbs catalyst **12** is a convenient strategy to prepare unsaturated macrocyclic lactones in a short sequence. Although isomerization is usually regarded as a disadvantage of RCM, it can be used to allow fast access to mass spectra of several isomers, helpful for the structure elucidation of natural compounds, e.g., in chemical ecology or fragrance research. The mass spectral fragmentation of macrolides differs markedly from that of open-chain esters, because initial bond cleavage often does not lead to the release of an uncharged radical, but to the formation of a distonic cationic radical prone to further fragmentation. The analysis of the fragmentation led to a rationale for the determination of the bound bond position in unsaturated macrolides. The identification of **1** and **5** in the gular gland of *H. cinnamomeoventris* and other species underlines the importance of macrolides for the chemical ecology of hyperoliid and mantellid frogs. On the contrary, other compounds such as the terpenes commonly found in hyperoliids remain largely unknown. Their identification and synthesis are a priority and would constitute a major step towards a biological evaluation of the gland secretion and its compounds to understand their real function in the behavior of the frogs. Although no experimental evidence has been obtained so far, the close association of the innervation of the

gland with the mating period, its location and use during calling, and its male-specific occurrence strongly hint to a function of **1** and **5** as signaling compounds and a role in the chemical ecology of this species.

Supporting Information

Supporting Information File 1

Experimental procedures, mass spectra of macrolides, alternative fragmentation pathway, enantiomer separation by GC–MS, ^1H and ^{13}C NMR spectra.
[<http://www.beilstein-journals.org/bjoc/content/supplementary/1860-5397-12-269-S1.pdf>]

Acknowledgements

Work in Madagascar was carried out in the framework of a collaboration accord between TU Braunschweig and the Université d'Antananarivo, Département de Biologie Animale. We are grateful to the Malagasy authorities for research and export permits. Specimen collection in Rwanda was conducted within a collaboration between the University of Vienna and the University of Koblenz-Landau, Germany. We would like to particularly thank M. Dehling and P. M. Maier for their support. Specimen collection and export was authorized by the Rwanda Development Board. The Cameroon Ministry of Forests and Wildlife (MINFOP) and Ministry of Scientific Research and Innovation (MINRESI) provided necessary permits for conducting research and exportation in Cameroon.

Our study received financial support from the Deutsche Forschungsgemeinschaft (DFG Schu984/10-1, MM, PP, SS), the Austrian Science Fund (FWF): P25612 (IS, WH), and the US National Science Foundation (DEB-1202609, DCB), and under the approval of the Institutional Animal Care and Use Committee (2014-2) at the California Academy of Sciences.

References

- Schulz, S.; Hölling, S. *Nat. Prod. Rep.* **2015**, *32*, 1042–1066. doi:10.1039/C5NP00006H
- Hölling, S.; Haberlag, B.; Tamm, M.; Collatz, J.; Mack, P.; Steidle, J. L. M.; Vences, M.; Schulz, S. *Chem. – Eur. J.* **2014**, *20*, 3183–3191. doi:10.1002/chem.201304414
- Poth, D.; Peram, P. S.; Vences, M.; Schulz, S. *J. Nat. Prod.* **2013**, *76*, 1548–1558. doi:10.1021/np400131q
- Poth, D.; Wollenberg, K. C.; Vences, M.; Schulz, S. *Angew. Chem., Int. Ed.* **2012**, *51*, 2187–2190. doi:10.1002/anie.201106592
- Stamberger, I.; Poth, D.; Peram, P. S.; Schulz, S.; Vences, M.; Knudsen, J.; Barej, M. F.; Rödel, M.-O.; Walzl, M.; Hödl, W. *Biol. J. Linn. Soc.* **2013**, *110*, 828–838. doi:10.1111/bj.12167
- Ando, T.; Yamakawa, R. *TrAC, Trends Anal. Chem.* **2011**, *30*, 990–1002. doi:10.1016/j.trac.2011.03.010

7. Buser, H. R.; Am, H.; Guerin, P.; Rauscher, S. *Anal. Chem.* **1983**, *55*, 818–822. doi:10.1021/ac00257a003
8. Jham, G. N.; Attygalle, A. B.; Meinwald, J. *J. Chromatogr. A* **2005**, *1077*, 57–67. doi:10.1016/j.chroma.2005.01.073
9. Kroiss, J.; Svatoš, A.; Kaltenpoth, M. *J. Chem. Ecol.* **2011**, *37*, 420–427. doi:10.1007/s10886-011-9933-4
10. Francke, W. *Chemoecology* **2010**, *20*, 163–169. doi:10.1007/s00049-010-0048-0
11. Millar, J. G.; Pierce, H. D., Jr.; Pierce, A. M.; Oehlschlager, A. C.; Borden, J. H.; Barak, A. V. *J. Chem. Ecol.* **1985**, *11*, 1053–1070. doi:10.1007/BF01020675
12. Oehlschlager, A. C.; King, G. G. S.; Pierce, H. D., Jr.; Pierce, A. M.; Slessor, K. N.; Millar, J. G.; Borden, J. H. *J. Chem. Ecol.* **1987**, *13*, 1543–1554. doi:10.1007/BF01012296
13. Millar, J. G.; Pierce, H. D., Jr.; Pierce, A. M.; Oehlschlager, A. C.; Borden, J. H. *J. Chem. Ecol.* **1985**, *11*, 1071–1081. doi:10.1007/BF01020676
14. Vanderwel, D.; Pierce, H. D., Jr.; Oehlschlager, A. C.; Borden, J. H.; Pierce, A. M. *Insect Biochem.* **1990**, *20*, 567–572. doi:10.1016/0020-1790(90)90068-6
15. Vanderwel, D.; Johnston, B.; Oehlschlager, A. C. *Insect Biochem. Mol. Biol.* **1992**, *22*, 875–883. doi:10.1016/0965-1748(92)90114-T
16. Millar, J. G.; Oehlschlager, A. C.; Wong, J. W. *J. Org. Chem.* **1983**, *48*, 4404–4407. doi:10.1021/jo00171a055
17. Sakai, T.; Hamamoto, H.; Mori, K. *Agric. Biol. Chem.* **1986**, *50*, 1621–1627. doi:10.1246/bcsj.50.1621
18. Naoshima, Y.; Nakamura, A.; Munakata, Y.; Kamezawa, M.; Tachibana, H. *Bull. Chem. Soc. Jpn.* **1990**, *63*, 1263–1265. doi:10.1246/bcsj.63.1263
19. Hamada, T.; Daikai, K.; Irie, R.; Katsuki, T. *Synlett* **1995**, 407–408. doi:10.1055/s-1995-5001
20. Mori, K.; Tomioka, H. *Liebigs Ann. Chem.* **1992**, 1011–1017. doi:10.1002/ljac.1992199201167
21. Fürstner, A.; Langemann, K. *J. Org. Chem.* **1996**, *61*, 3942–3943. doi:10.1021/jo960733v
22. Breinbauer, R.; Jacobsen, E. N. *Angew. Chem., Int. Ed.* **2000**, *39*, 3604–3607. doi:10.1002/1522-3773(20001016)39:20<3604::AID-ANIE3604>3.0.CO;2-9
23. Schaus, S. E.; Brandes, B. D.; Larrow, J. F.; Tokunaga, M.; Hansen, K. B.; Gould, A. E.; Furrow, M. E.; Jacobsen, E. N. *J. Am. Chem. Soc.* **2002**, *124*, 1307–1315. doi:10.1021/ja016737i
24. Neises, B.; Steglich, W. *Angew. Chem., Int. Ed. Engl.* **1978**, *17*, 522–524. doi:10.1002/anie.197805221
25. Searles, S., Jr.; Pollart, K. A.; Lutz, E. F. *J. Am. Chem. Soc.* **1957**, *79*, 948–951. doi:10.1021/ja01561a046
26. Rost, D.; Porta, M.; Gessler, S.; Bleichert, S. *Tetrahedron Lett.* **2008**, *49*, 5968–5971. doi:10.1016/j.tetlet.2008.07.161
27. Hong, S. H.; Sanders, D. P.; Lee, C. W.; Grubbs, R. H. *J. Am. Chem. Soc.* **2005**, *127*, 17160–17161. doi:10.1021/ja052939w
28. Maynard, H. D.; Grubbs, R. H. *Tetrahedron Lett.* **1999**, *40*, 4137–4140. doi:10.1016/S0040-4039(99)00726-1
29. Herbert, M. B.; Grubbs, R. H. *Angew. Chem., Int. Ed.* **2015**, *54*, 5018–5024. doi:10.1002/anie.201411588
30. Marx, V. M.; Herbert, M. B.; Keitz, B. K.; Grubbs, R. H. *J. Am. Chem. Soc.* **2013**, *135*, 94–97. doi:10.1021/ja311241q
31. Schulz, S.; Yildizhan, S.; Stritzke, K.; Estrada, C.; Gilbert, L. E. *Org. Biomol. Chem.* **2007**, *5*, 3434–3441. doi:10.1039/b710284d
32. Moore, B. P.; Brown, W. V. *Aust. J. Chem.* **1976**, *29*, 1365–1374. doi:10.1071/CH9761365
33. Wong, J. W.; Verigin, V.; Oehlschlager, A. C.; Borden, J. H.; Pierce, H. D., Jr.; Pierce, A. M.; Chong, L. *J. Chem. Ecol.* **1983**, *9*, 451–474. doi:10.1007/BF00990219
34. Fürstner, A.; Thiel, O. R.; Ackermann, L.; Schanz, H.-J.; Nolan, S. P. *J. Org. Chem.* **2000**, *65*, 2204–2207. doi:10.1021/jo9918504
35. Lehman, S. E., Jr.; Schwendeman, J. E.; O'Donnell, P. M.; Wagener, K. B. *Inorg. Chim. Acta* **2003**, *345*, 190–198. doi:10.1016/S0020-1693(02)01307-5
36. van Lierop, B. J.; Lummis, J. A. M.; Fogg, D. E. Ring-closing metathesis. In *Olefin Metathesis: Theory and Practice*; Grela, K., Ed.; Wiley: Hoboken, 2014; pp 85–152. doi:10.1002/9781118711613.ch3
37. Courchay, F. C.; Sworen, J. C.; Wagener, K. B. *Macromolecules* **2003**, *36*, 8231–8239. doi:10.1021/ma0302964
38. Petkovska, V. I.; Hopkins, T. E.; Powell, D. H.; Wagener, K. B. *Macromolecules* **2005**, *38*, 5878–5885. doi:10.1021/ma050480k

License and Terms

This is an Open Access article under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

The license is subject to the *Beilstein Journal of Organic Chemistry* terms and conditions: (<http://www.beilstein-journals.org/bjoc>)

The definitive version of this article is the electronic one which can be found at:
doi:10.3762/bjoc.12.269

