Studies on Neotropical Fauna and Environment, April 2005; 40(1): 23 – 28

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ORIGINAL ARTICLE

Stomach-flushing for diet analysis in anurans: an improved protocol evaluated in a case study in *Araucaria* forests, southern Brazil*

MIRCO SOLÉ[1,2], OLAF BECKMANN[1,2], BIRGIT PELZ[1,2], AXEL KWET[1,2,3] & WOLF ENGELS[1,2]

[1]*Laboratory of Biological Research (LPB), PUCRS, Porto Alegre, Brazil,* [2]*Zoological Institute, University of Tübingen, Germany, and* [3]*Natural History Museum, Department of Zoology, Stuttgart, Germany*

(Received 15 July 2004; accepted 28 September 2004)

Abstract
Stomach-flushing is a gentle treatment in analysing the diet of vertebrates. We applied this method in the study of seasonal nutrition of anurans of 15 species at an *Araucaria* forest habitat on the Serra Geral of Rio Grande do Sul, Brazil. Over 500 frogs and toads of different body size were handled in order to evaluate an improved protocol without narcosis using soft infusion tubes in order to avoid negative effects of the flushing procedure. The specimens were treated soon after capture and then returned to the sampling sites. Our results are discussed with reference to the few studies on stomach flushing in amphibians. The improved technique is recommended to avoid killing of numerous animals for nutritional studies, in particular because of the worldwide threatening of amphibian populations.

Keywords: *Diet, stomach-flushing, Amphibia, Anura, biodiversity, Araucaria forest, Brazil*

Introduction

A major topic in our long-term studies on amphibians on the *Araucaria* plateau of Rio Grande do Sul, Brazil (Kwet, 2001) is the role of anurans as predators and prey in the ecosystem of *Araucaria* rain forests and surrounding habitats (Engels & Heinle, 2003). About 39 species of amphibians have been recorded so far in or near the mountain forest reserve Pró-Mata on the Serra Geral, some of them in high abundance (Kwet, 2004). Dietary patterns in anurans have been commonly analysed by sacrificing the animals, dissecting their stomach and determining the prey remnants (Toft, 1981; Das & Coe, 1994; Flowers & Graves, 1995; Evans & Lampo, 1996; Lima, 1998; Löw & Török, 1998; Anderson et al., 1999; Daza-Vaca & Castro-Herrera, 1999; Peltzer & Lajmanovich, 1999; Van Sluys et al., 2001). In order to avoid killing a representative number of specimens, alternative methods were developed of which the stomach-flushing approach is particularly simple and effective. Originally applied to salamanders (Fraser, 1976) and freshwater turtles (Legler, 1977), the technique was also successfully used in lizards and frogs (Legler & Sullivan, 1979). The treatment was mostly done after narcotising the specimens but this sometimes caused mortality (Joly, 1987). Because amphibians are declining worldwide (Wake, 1991), any research affecting the biodiversity should be scheduled with care.

We used stomach-flushing over four years in a study of the diet of 15 anuran species at the Pró-Mata reserve. Our experience from well over 500 treated frogs and toads resulted in an improved protocol for flushing without anaesthesia of small as well as large individuals, representing all adult stages.

Material and methods

Study area

Our study site was the forest reserve Centro de Pesquisas e Conservação da Natureza Pró-Mata, located at altitudes of about 900 m a. s. l. on the

Correspondence: M. Solé, Zoologisches Institut der Universität Tübingen, Auf der Morgenstelle 28, D – 72076 Tübingen, Germany. E-mail: mirco.sole@uni-tuebingen.de

* Part of the projects DLR-BMBF 01LT0011/7 'Araucaria forest' and DLR/IB-FAPERGS 'Subsidios para o diagnóstico ambiental do Planalto das Araucárias'.

ISSN 0165-0521 print/ISSN 1744-5140 online
DOI: 10.1080/01650520400025704

Serra Geral in Rio Grande do Sul, Brazil, approximately 150 km northeast of Porto Alegre. The site has a humid subtropical climate.

Sample of anurans

The field studies were carried out from 1999 to 2003. Frogs and toads belonging to five families were collected manually in their habitats and taken immediately to the laboratory for flushing (Table I). A total sample of 583 individuals was stomach-flushed. Most of them were returned to place of capture soon after the procedure, except 15 *Physalaemus lisei*, 4 *Leptodactylus plaumanni* and 10 *Pseudis cardosoi* which were kept in the laboratory for one month and observed for possible harm by the treatment.

Flushing technique

Flushing should be applied as soon as possible after capturing anurans, in order to precede digestion (Secor & Faulkner, 2002). We flushed all animals within three hours of capture. The following equipment (Figure 1) is required for anuran stomach-flushing:

Spatula, forceps, two syringes with thread (20 ml for small, 60 ml for large frogs), infusion tube of soft material (silicon), in particular for small individuals in order to avoid perforations of oesophagus and stomach, with thread, sieve, small airtight vials, two vessels, 70% ethanol. The water for flushing should be taken from the ponds where the anurans were captured and used after filtration. For direct developing animals as the frogs of the genus *Eleutherodactylus*, spring water should be used.

Description of the procedure:

The flushing is done without any anaesthetising and with careful handling of the animals.

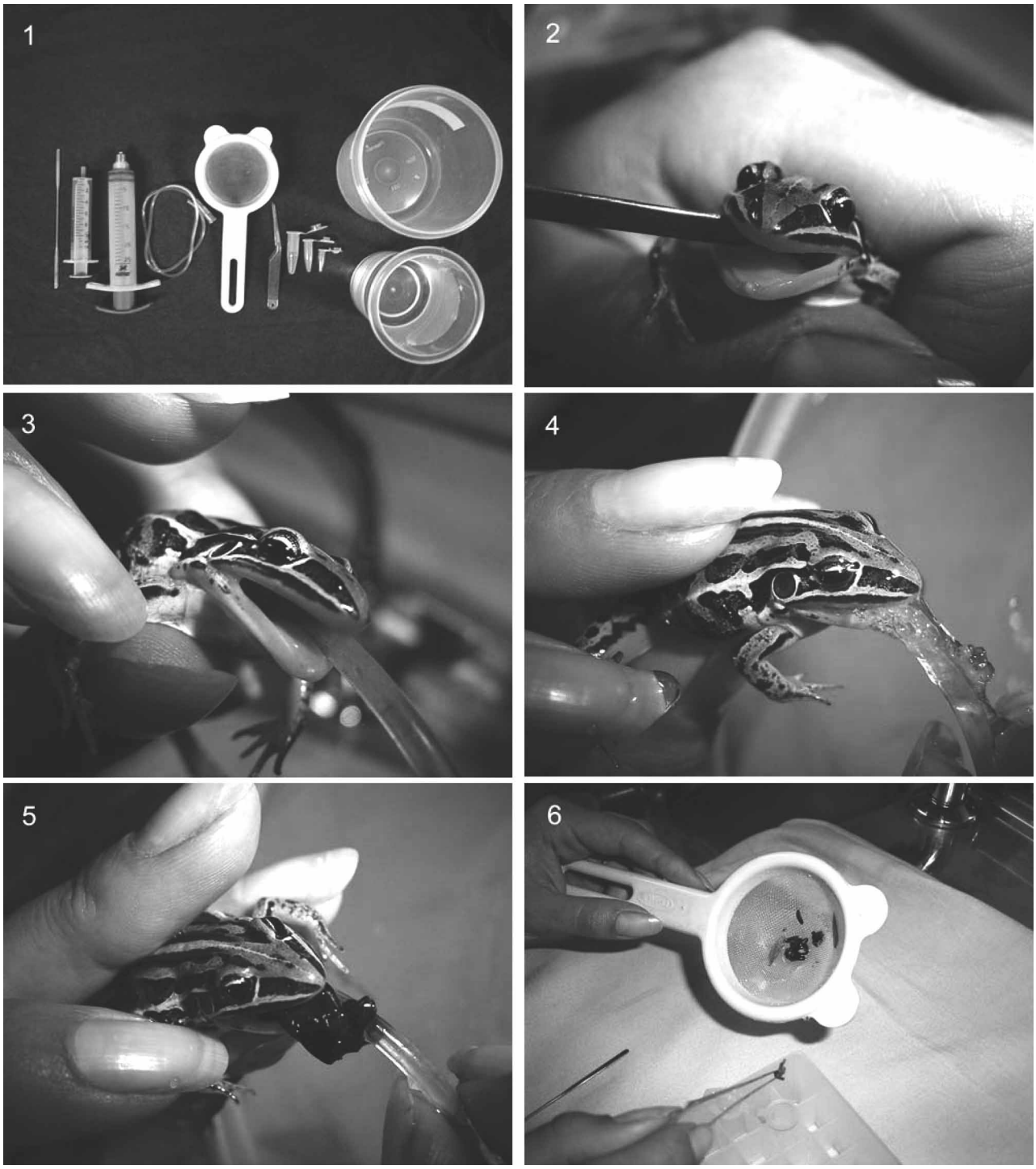
Step 1: The anuran can be held safely by fixing the forelimbs with one hand (Figure 2).
Step 2: With the animal in one hand and the prepared syringe (water-filled, tube attached) in the other, the mouth is opened with the help of a spatula. As both hands are in use, the spatula has to be held with the mouth or must be fixed in the ground. The tube of the syringe must be introduced through the oesophagus into the stomach. The pyloric end of the stomach can normally be felt (Figure 3).
Step 3: The entire content of the syringe is flushed into the stomach and any content that is forced out is collected in the vessel (Figures 4 and 5). The empty syringe should be separated from the tube, refilled with water and connected to the tube again to repeat the flushing procedure. This procedure should be repeated as long as stomach contents are forced out. When no more stomach content appears, the animal should be flushed one last time.

The water with stomach content is then decanted into a sieve. The items are picked up with forceps and fixed in 70% ethanol in a vial. For smaller specimens, 6 μm gauze is used instead of the sieve. The stomach content can directly be washed with 70% ethanol from the gauze into a small vial.

The subsequent immediate release into their home habitat ensures that the current activity of the treated specimens is not essentially disturbed by the stomach-flushing.

Table I. Size range and survival of anuran specimens that were stomach-flushed. Minimum and maximum size data correspond to adults and are taken from Kwet and Di-Bernardo (1999).

Family	Species	SVL mm	Flushed	Perished	Survived
Bufonidae	*Bufo ictericus*	100 – 140	78	1	77
Hylidae	*Hyla faber*	85 – 100	50	0	50
	Hyla minuta	21 – 28	50	0	50
	Hyla pulchella	33 – 47	50	0	50
	Scinax granulatus	34 – 43	50	1	49
	Scinax perereca	36 – 46	50	0	50
Leptodactylidae	*Eleutherodactylus* cf. *guentheri*	21 – 38	4	0	4
	Hylodes meridionalis	35 – 50	7	6	1
	Leptodactylus plaumanni	35 – 46	43	0	43
	Leptodactylus ocellatus	90 – 120	50	0	50
	Physalaemus cuvieri	24 – 32	8	0	8
	Physalaemus lisei	21 – 33	57	0	57
Microhylidae	*Elachistocleis bicolor*	20 – 45	16	0	16
	Elachistocleis erythrogaster	29 – 38	10	0	10
Pseudidae	*Pseudis cardosoi*	34 – 43	60	0	60
	Total		**583**	**8**	**575**



Figures 1 – 6. Flushing technique. (1) Equipment needed for stomach-flushing. (2) Immobilisation of the frog (*Leptodactylus plaumanni*) and insertion of the spatula to open the mouth. (3) Frog with tube entering the mouth (and reaching the stomach). (4) Frog being stomach-flushed. (5) Stomach contents pouring out of the mouth after flushing. (6) Sieve with stomach content to be fixed and stored.

Results

Stomach content obtained by flushing

Because anurans were stomach-flushed within three hours after capture, few of the items were totally intact, most were partially digested. However, parts with heavily sclerotised cuticle remained undigested so that single wings, heads, thorax and abdominal segments of arthropods allowed an identification of the item, at least to order level.

The presence or absence of animal food, which could be recovered through stomach-flushing, varied

depending on the analysed anuran taxa. In some species all flushed animals had stomach contents. This was the case for the leptodactylid frogs *Eleutherodactylus* cf. *guentheri*, with small arthropods in the stomach, *Hylodes meridionalis,* with mainly dipterans and coleopterans as stomach content, *Leptodactylus ocellatus*, preying on large coleopterans, spiders and other anurans, and *Physalaemus cuvieri*, with mostly small arthropods in the stomach and for the microhylids *Elachistocleis bicolor* and *E. erythrogaster*, both feeding exclusively on ants and termites. In 90% of the *Pseudis cardosoi*, food items were recovered, consisting mostly of coleopterans, dragonfly nymphs and hymenopterans. The animals with empty stomachs were captured during cold nights and therefore were probably not hunting. 88% of the *Physalaemus lisei* revealed stomach contents consisting mainly of ants, dipterans and spiders. From 77% of the *Leptodactylus plaumanni* food was recovered, consisting mostly of coleopterans and ants. Only half of the *Bufo ictericus* had food remnants, with coleopterans, ants and diplopods being the most frequent taxa. The other half were males with empty stomachs caught at a pond at night while calling. Male *Bufo ictericus* seem to stop feeding during courtship. Something similar seems to happen with the tree frogs: of the five species examined only *Hyla pulchella* revealed a high number of animals with food items in their stomachs, mainly being coleopterans, lepidopterans and hymenopterans. Of the two examined species of the genus *Scinax* only about 35% had stomach contents, those being mostly homopterans, coleopterans and mites for *Scinax granulatus* and spiders and blattarians for *Scinax perereca*. Nearly all smith-frogs (*Hyla faber*) had empty stomachs. The few frogs with stomach contents had mostly preyed on opilionids and anurans.

The largest prey items were a male *Scinax granulatus* (36.2 mm SVL) eaten by a male *Hyla faber* (95.3 mm SVL), a mygalomorph spider with a prosoma measuring 20.6 mm eaten by a female *Leptodactylus ocellatus* (69 mm SVL) and a 48.2 mm large dragonfly nymph captured by another female *L. ocellatus* (78.6 mm SVL). The smallest items were collembolans and mites.

Reliability of the flushing technique

Flushing is a reliable method to recover stomach contents. In most of the anurans the contents were recovered completely after the second flushing and only seldom did the procedure need to be repeated (maximum of six times) before all contents were recovered. If individuals are captured during or shortly after their foraging activity and flushed no longer than three hours later most of the stomach contents will be undigested and can subsequently be identified without problems.

Survival of the flushed anurans

Nearly all flushed anurans survived the procedure without apparent problems. Of eight injured specimens, one *Bufo ictericus* and one *Scinax granulatus* died because their gut was punctured with the tube and the lungs were filled with water. Six *Hylodes meridionalis* perished without apparent harm, showing strong muscular contractions in the hind limbs shortly before death. These specimens, analysed in the initial phase of our study, had been flushed with tap water. Without our knowledge this had been treated with high doses of chlorine and this fact presumably caused the death of the frogs. Therefore, we decided to use only pond or spring water to flush the stomachs. Altogether the total mortality in our experiments was about 1% only.

Twenty-nine stomach-flushed frogs were kept in captivity for one month and accepted termites as food within the next two hours. After one month, they all seemed to be healthy and were released back to the pond where they had been collected.

Discussion

Evaluation of the improved stomach-flushing technique

Our flushing protocol differs from previous methods. The whole procedure can be conducted by a single person. No anaesthetic is needed which caused mortality in previous studies (Legler & Sullivan, 1979). The use of a soft silicone infusion tube prevents lesions of the gut tissues. Even small and recently metamorphosised frogs with an SVL of only 21 mm can be flushed without problems. The immediate return to the place of capture prevents behavioural disturbance, especially during the period of reproduction.

Previous experience with flushing of amphibians

Since introduction of the stomach-flushing method (Legler & Sullivan, 1979) with brevital sodium to anesthetise animals, the technique was modified in subsequent studies. Working with ranid frogs, Leclerc and Courtois (1993) used tricaine methane sulphonate as a narcotic in order to reduce the mortality. These authors killed some flushed frogs to test if food remained in the stomach and found that less than 1% of the total food volume was not removed. Stomach-flushing was used by Ovaska (1991) to analyse the diet of *Eleutherodactylus johnstonei* in Barbados and by Díaz-Páez and Ortiz (2003) to study the feeding habits of the leptodacty-

lid frog *Pleurodema thaul* in Chile. Lamoureux et al. (2002) used stomach-flushing in order to study premigratory autumn foraging forays in the green frog, *Rana clamitans*. Tocque et al. (1995) studied the prey range of the desert toad (*Scaphiopus couchii*) but did not precisely describe their stomach-flushing method. In some European anurans this technique was used to determine the minimum sample size for estimating diet diversity (Kovács & Török, 1997). Gittins (1987) compared the results of stomach-flushing in common toads (*Bufo bufo*) with dissections of road casualties and obtained similar results. Patto (1998) adapted stomach-flushing to small frogs working with *Hylodes asper*. He anaesthetised them for a few seconds in a plastic bag containing cotton soaked with chloroform before flushing the stomach. He subsequently dissected six animals and detected no food remains. Of 97 toe-clipped and released frogs 13 were recaptured once and seven twice. They had food in their stomachs, suggesting that they had not been injured by the procedure and had resumed normal feeding activities. Patto also tested the method on an abundant invader species, *Leptodactylus fuscus*. Of the 20 flushed and the dissected frogs, none had any more food items in the stomach. He recommended the stomach-flushing approach in dietary studies of rare, endemic, or protected species and for research on Amphibian population dynamics.

Conclusions

Our improved stomach-flushing protocol was tested with small (SVL 21 mm) and large anurans (SVL 140 mm). The low proportion of injured individuals showed that this method works well if correctly applied. The fact that 29 stomach flushed frogs survived well and fed normally in captivity suggests that they suffered no inner lesions or other disturbances during the procedure. Corresponding results were obtained in the first application of our method for dietary studies in the microhylid frog *Elachistocleis bicolor* (Solé et al., 2002). Therefore, we can strongly recommend using stomach-flushing in research on the diet of anurans. We agree with the statement of the Northern Territory University Animal Experimentation Ethics Committee that 'there is now no justification for killing animals solely in order to obtain stomach samples' (Northern Territory University, 2000). In addition, the programs of monitoring biodiversity and the nutritional requirements of vertebrate species should also consider these aspects.

Acknowledgments

This research was performed with the long-term cooperation between the University of Tübingen and the PUCRS, Porto Alegre, RS, Brazil. We would like to thank the IMA, in particular Jorge A. Villwock, and the MCT, especially Marcos Di-Bernardo, for valuable support. We are grateful to Paul Müller, David Wake and Anne Zillikens for constructive comments on the manuscript.

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