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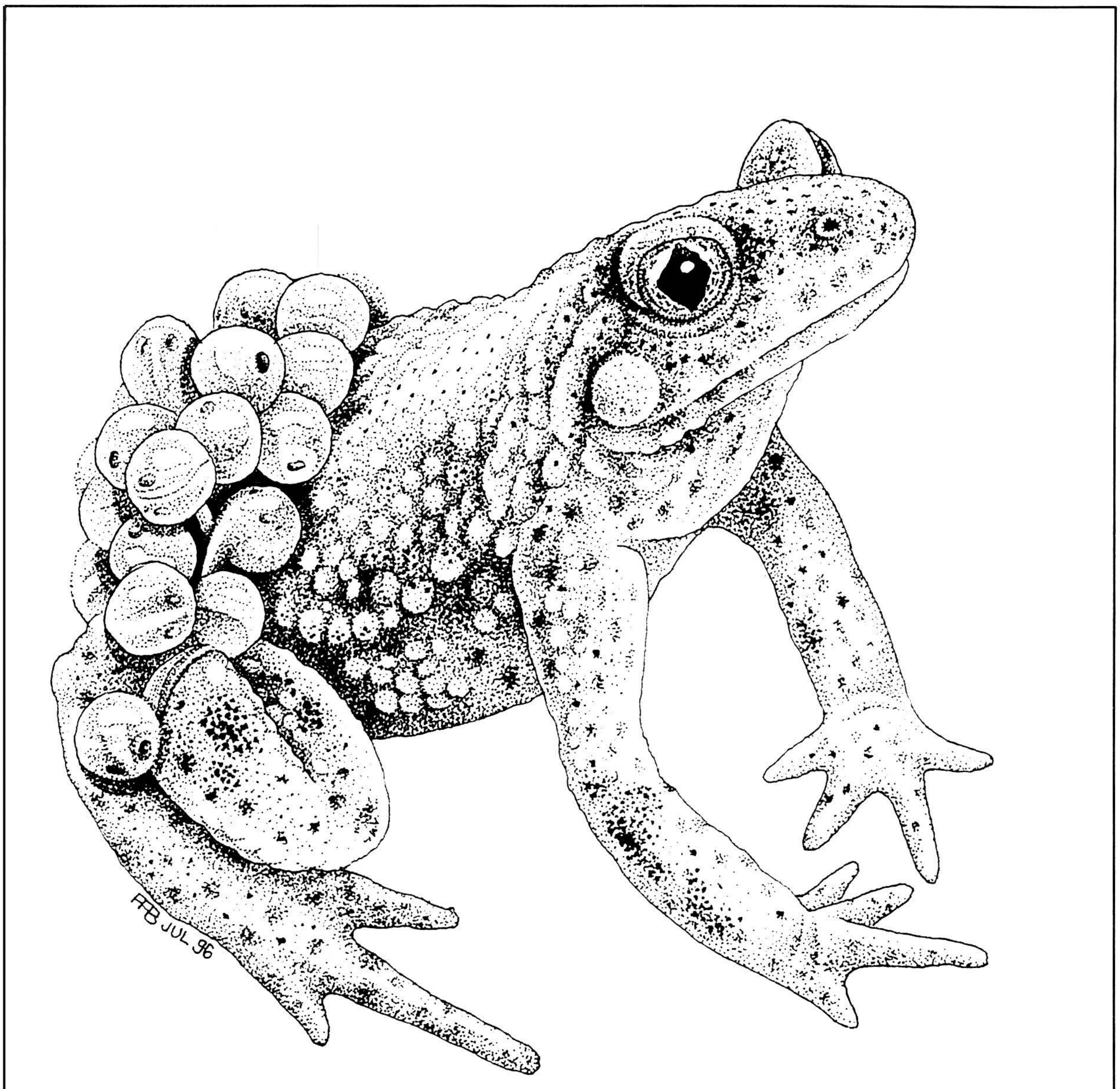

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DISCRIMINANT FUNCTIONS FOR SEX IDENTIFICATION IN TWO MIDWIFE TOADS (*ALYTES OBSTETRICANS* AND *A. CISTERNASII*)

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Determining the sex of midwife toads in the field is not easy. Non-pregnant females and males not tending clutches are difficult to sex without dissection. We provide a method to determine the sex of individuals based on the study of linear variables. Fifteen morphological variables were measured from samples of two species of midwife toad in the Iberian Peninsula (*Alytes obstetricans* and *Alytes cisternasii*). Some variables, corrected for the size of the animal, show significant differences between sexes. A discriminant analysis between the sexes in both species shows a high power for discrimination (95% in *A. obstetricans* and 97.6% in *A. cisternasii*). The significant variables in *A. obstetricans* were: snout-urostyle length, distance between the nostrils, distance between the anterior end of the middle metacarpal tubercle and the tip of the third finger, and distance from elbow to third finger tip. The significant variables in *A. cisternasii* were: head width, jaw bottom length, vertical diameter of the tympanum, distance between the nostrils, and tibia-fibula length.

INTRODUCTION

As Darwin (1871) pointed out: "It is surprising that frogs and toads should not have acquired more strongly-marked sexual differences; for though cold-blooded, their passions are strong". Adult midwife toads (genus *Alytes*) are a good example of similarity between the sexes because they lack secondary sexual characters, either permanent or seasonal. During the mating season males differ from females because they have an advertisement call which is louder than the calls of females, however call intensity is not an adequate discriminating characteristic beacuse it is extremely difficult to observe individuals calling (Heinzmann, 1970; Márquez & Verrell, 1991). Midwife toads have male parental care of the eggs on land. During the brooding period the male carries a string of eggs entwined around his ankles and hence individuals carrying eggs are males. Similarly, females that contain mature oviductal eggs can be identified as such by observing the eggs through the transparent skin of their lower abdomen. However, outside these special situations, it is virtually impossible to tell apart a silent male not tending eggs from a female without oviductal eggs.

Crespo (1982) studied the differences between the sexes for 23 osteological variables and found that in *A. obstetricans boscai* none of the variables were significantly different between the sexes while in *A. cisternasii* only one of 23 variables yielded a significant difference. In general, the larger relative sizes of some segments of the limbs of males and the larger absolute body size of the females (see also Márquez, Esteban & Castanet, 1996) are the only apparent discriminating characters between the sexes. García-París (1992) studied sexual dimorphism in four populations of *Alytes* and found low levels of sexual dimorphism. He found that head width was the only variable that presented relatively marked dimorphism across populations. In a single population of *A. obstetricans*, the variables that presented significant differences between the sexes were head width and minimun distance of the eye to the nostril.

Sexing adult midwife toads in the field may be essential for ethological or ecological studies, and particularly for conservation-related studies (García-París, 1992). Therefore, it is of interest to develop a method that allows the determination of the sex of adult individuals with a high degree of accuracy. Discriminant analysis is a widely used multivariate technique (e.g. Van Vark & Schaafsma, 1992) and is a method of predicting some level of a one-way classification based on known values of the responses. The technique is based on how close a set of measurement variables are to the multivariate means of the levels being predicted. To the best of our knowledge, this technique has not been used for sexing anurans indistinguishable in the field, but it is regularly used to solve taxonomic problems in complex groups (e.g. Heyer, 1978).

MATERIAL AND METHODS

Two populations of midwife toads were studied, one of *A. obstetricans* and one of *A. cisternasii*. The population of *A. obstetricans* occurred near the shores of Peñalara's lake, an alpine lake (2000 m a.s.l) in the Sierra de Guadarrama (province of Madrid). Bioacoustical data from this population (Márquez & Bosch, 1995) suggests that it may be ascribed to the newly described subspecies *A. o. almogavarii* (Arntzen

TABLE 1. Morphological variables measured and abbreviations.

SVL	Snout-urostyle length.
HW	Head width (between tympanae).
JL	Jaw bottom length.
ED	Minimum distance between eyes.
HT	Horizontal diameter of tympanum.
VT	Vertical diameter of tympanum.
EW	Eye width.
ND	Distance between nostrils.
END	Minimum distance of between eye and nostril.
TFL	Tibia-fibula length.
HLL	Hind limb length.
TL	Distance between the tarsal tubercle and the tip of the third toe.
FTL	Distance between anterior end of middle metatarsal tubercle and tip of 3rd finger.
FL	Length of 3rd finger.
EFD	Distance from elbow to 3rd finger tip.

& García-París, 1995) and not to *A. o. boscai* as was previously thought. The population of *A. cisternasii* was studied near the Roman dam of Proserpina, a site in a live oak forest, at 235 m a.s.l, located 5 km north of Mérida (Badajoz), the species' *terra typica*.

A total of twenty males and twenty females of *A. obstetricans* and twenty one males and twenty females of *A. cisternasii* were used for the measurements. In all cases, measurements were taken from living individuals that were freed immediately after being measured. Measurements were taken with a digital caliper Mitutoyo CD-15 (precision, 0.01 mm) or with an analog caliper AEMM (precision, 0.05 mm). The sex of all individuals measured was known. Only males observed calling or males captured after being observed releasing their egg masses in the water were used. Similarly, only gravid females with developed eggs observable through the transparent skin of the lower abdomen were measured.

TABLE 2. Mean, standard deviation and ranges for each sex, in the two species studied, and Student *t* test for differences between sexes (M, males and F, females). See Table 1 for abbreviations.

		Alytes obstetricans							*Alytes cisternasii*						
		n	Mean	SD	Min.	Max.	*t*	*P*	*n*	Mean	SD	Min.	Max.	*t*	*P*
SVL	F	20	51.60	3.04	43.40	58.00	4.94	0.0001	20	39.83	2.82	36.00	46.00	2.69	0.0105
	M	20	46.15	3.89	38.00	52.00			21	37.75	2.08	33.00	41.00		
HW	F	20	15.77	0.74	14.40	17.00	3.91	0.0004	20	13.68	0.65	13.00	15.10	1.63	0.1112
	M	20	14.61	1.10	12.95	16.55			21	13.40	0.46	12.45	14.25		
JL	F	20	15.06	0.87	12.60	16.70	3.83	0.0005	20	10.77	0.63	9.55	11.85	3.13	0.0033
	M	20	13.81	1.17	11.67	16.15			21	10.17	0.61	8.85	11.00		
ED	F	20	4.11	0.28	3.60	4.65	0.13	0.8972	20	4.08	0.23	3.70	4.50	0.35	0.7287
	M	20	4.10	0.31	3.54	4.60			21	4.11	0.22	3.70	4.50		
HT	F	20	3.74	0.38	2.60	4.35	0.78	0.4426	20	3.15	0.25	2.70	3.80	1.62	0.1130
	M	20	3.65	0.36	2.73	4.30			21	3.30	0.33	2.65	4.00		
VT	F	20	3.81	0.30	2.90	4.10	1.27	0.2114	20	2.99	0.30	2.40	3.45	2.67	0.0111
	M	20	3.67	0.41	2.62	4.30			21	3.22	0.25	2.65	3.55		
EW	F	20	5.04	0.29	4.40	5.82	3.49	0.0013	20	3.97	0.21	3.60	4.55	0.54	0.5946
	M	20	4.61	0.47	3.75	5.55			21	4.01	0.28	3.45	4.45		
ND	F	20	3.99	0.33	3.30	4.87	1.04	0.3068	20	3.44	0.16	3.15	3.75	3.11	0.0035
	M	20	3.88	0.34	3.40	4.44			21	3.26	0.19	2.95	3.80		
END	F	20	5.01	0.28	4.40	5.50	4.15	0.0002	20	3.87	0.21	3.50	4.20	1.81	0.0787
	M	20	4.53	0.44	3.64	5.10			21	3.75	0.21	3.45	4.25		
TFL	F	20	19.02	0.97	17.70	20.85	2.49	0.0171	20	13.16	0.75	12.20	14.85	1.27	0.2135
	M	20	17.99	1.58	14.84	19.85			21	13.44	0.66	12.25	14.70		
HLL	F	20	64.64	3.38	59.00	70.80	2.95	0.0005	20	46.09	3.30	40.90	53.45	0.49	0.6279
	M	20	60.56	5.17	51.21	67.30			21	46.54	2.62	40.45	51.60		
TL	F	20	15.71	1.17	13.40	17.65	1.98	0.0054	20	12.07	0.97	10.30	13.70	0.48	0.6370
	M	20	14.84	1.58	11.54	17.80			21	11.92	0.93	10.50	13.85		
FTL	F	20	7.86	0.40	6.90	8.49	1.23	0.2273	20	6.03	0.37	5.45	6.80	0.89	0.3782
	M	20	7.65	0.65	6.34	9.00			21	5.91	0.44	5.25	6.90		
FL	F	20	6.11	0.51	5.20	7.01	1.07	0.2929	20	3.73	0.29	3.35	4.45	1.27	0.2105
	M	20	5.93	0.51	4.59	6.80			21	3.86	0.38	3.15	4.60		
EFD	F	20	20.71	1.24	17.50	22.40	2.26	0.0299	20	15.44	0.87	13.60	16.95	1.34	0.1897
	M	20	19.69	1.62	16.64	22.30			21	15.84	1.03	14.10	18.05		

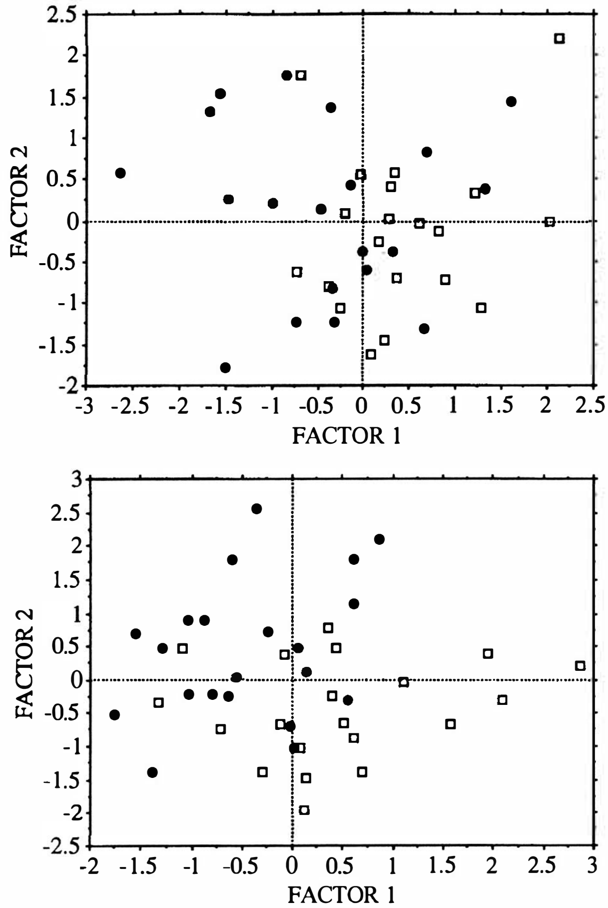

FIG. 1. Principal component analysis for *A. obstetricans* (top) and *A. cisternasii* (bottom). Females are represented by open squares, males by solid circles.

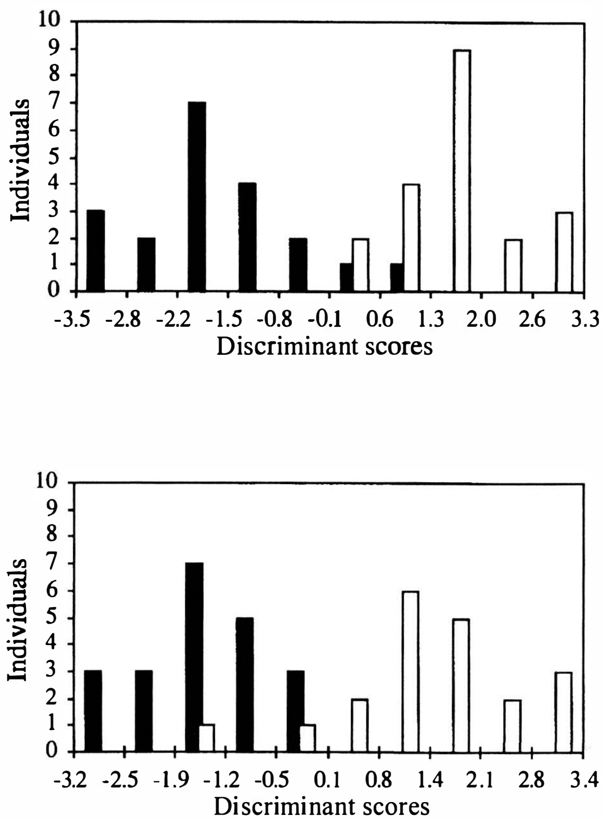

FIG. 2. Graphical representation of scores of discriminant analysis of sexes in *A. obstetricans* (top) and *A. cisternasii* (bottom). Females are represented by open bars, males by solid bars.

A total of 15 morphological variables were measured (Table 1). SVL was measured by pressing the animals flat against a ruler (ventral side). All the variables of bilaterally symmetric features were measured on the right side. Statistical analyses were performed with software SPSS 5.0, Statistica 4.1 and Statview 4.1. Stepwise discriminant analyses were performed with *P* in 0.05, and *P* out 0.10.

RESULTS

Sex specific mean, standard deviation and ranges of the variables measured are shown in Table 2 for both species. These uncorrected data show that, in most *A. obstetricans* measurements (9/15, 60%) females are significantly larger than males, while only four out of 15 variables (36%) showed significant differences in *A. cisternasii*. The data were log-transformed to correct for the allometric effect of continuous growth, and to minimize the differences between variances. For each species every one of these log-transformed variables was used in a linear regression with snout-vent length (SVL), and the residuals of these regressions were used to compare values between sexes.

After transformation, five out of 14 variables (36%) showed significant differences between sexes in *A. obstetricans* (HT, $t = -2.74$, $P = 0.0094$; ND, $t = -2.20$, $P = 0.0341$; TL, $t = -2.35$, $P = 0.0238$; FTL, $t = -3.06$, $P = 0.004$; EFD, $t = -3.69$, $P = 0.0007$), and six out of 14 variables (43%) in *A. cisternasii* (HT, $t = -3.20$, $P = 0.0027$; VT, $t = -3.59$, $P = 0.0009$; EW, $t = -2.31$, $P = 0.0263$; TFL, $t = -3.55$, $P = 0.001$; HLL, $t = -2.69$, $P = 0.0105$; EFD, $t = -3.01$, $P = 0.0045$). Only two of these variables (HT and EFD) showed significant differences in both species. A principal component analysis (Fig. 1) shows great similarity between the sexes in both species.

To obtain a system for classification of individuals of unknown sex, discriminant analyses were used. A stepwise discriminant analysis was applied to the data for each species to obtain discriminant functions that maximize the correct classification of individuals. The histograms of the scores of the discriminant functions are shown in Fig. 2. The discriminant function for sex in *A. obstetricans* is: 0.8782 x SVL - 2.0082 x ND - 1.4129 x FTL - 1.0611 x EFD - 2.6330, and the cut-off point is 0.0000. For *A. cisternasiii* the discriminant function is: 1.5344 x HW + 1.4455 x JL - 1.9878 x VT + 3.7914 x ND - 2.1746 TFL - 13.4771, and the cut-off point is 0.0368. If the value obtained in the function is above the cut-off point, the diagnosis will be female, and if it is below the cut-off point, the diagnosis will be

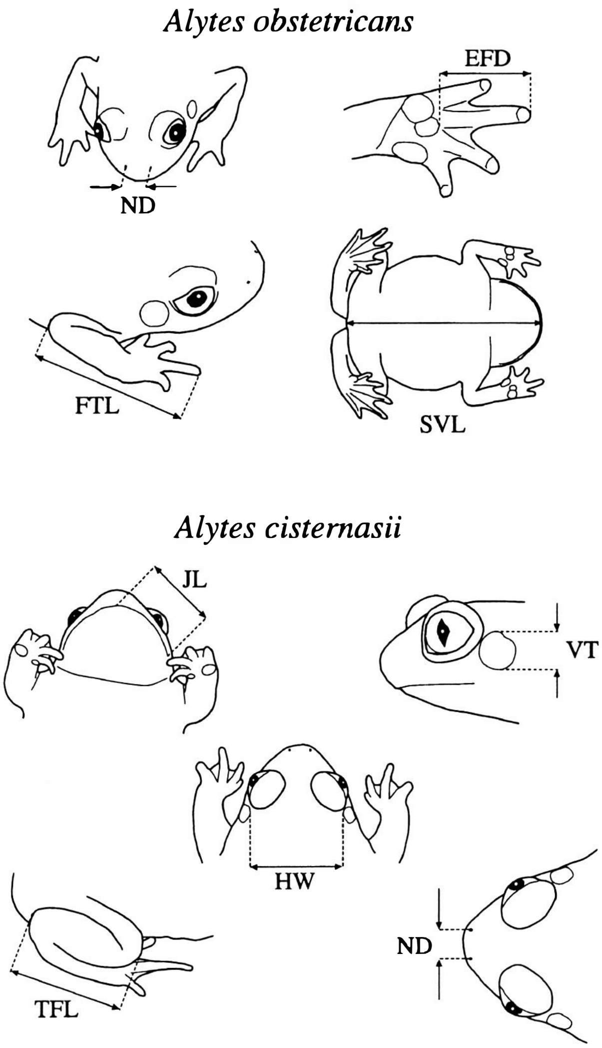

FIG. 3. Diagnostic measurement for each species.

male. For *A. obstetricans* only four variables were included in the function (SVL, ND, FTL, EFD), while for *A. cisternasii* five variables were included (HW, JL, VT, ND, TFL), Fig. 3. The discriminant function obtained for *A. obstetricans* classified correctly 95% of the individuals (100% of the females and 90% of the males), while the function obtained for *A. cisternasii* classifies correctly 97.56% of the individuals (95% of the females and 100% of the males).

Other discriminant analyses were performed with all the variables taken one at a time. In *A. obstetricans* the variable JL showed the highest discriminant power, classifying correctly 80% of the individuals (90% of the females and 70% of the males). For *A. cisternasii,* the variable with highest discriminant power was ND which correctly classified 83% of the cases (80% of the females and 86% of the males). For *A. obstetricans,* if 14.132 x JL - 107.131 > 12.959 x JL - 90.199, then the individual is classified as female; for *A. cisternasii,* if 111.062 x ND - 191.467 > 105.543 x ND - 172.930, then the individual is classified as female.

DISCUSSION

In *A. obstetricans,* females appear to be consistently larger than males, while in *A. cisternasii* the differences are less marked. It appears that measures reflecting relative tympanum size (HT, VT) are proportionately larger in males although the differences were not significant in three of four cases, in absolute values. This could be the result of the selective pressure imposed by the vocalisations on the auditory structure. Both males and females are supposed to have their auditory system tuned to the same frequency ranges (determined by the ranges of the advertisement calls of the males in their populations) and, given that tympanum size may have an effect on frequency tuning (Shofner & Feng, 1984), it follows that tympanae of males and females should have similar sizes. Since males have smaller body sizes, this would explain the difference observed in relative tympanum sizes. Similarly, relative forelimb lengths (EFD) are larger in males in both species, possibly reflecting selective pressures for amplexus ability (forelimbs). On the other hand, relative hindlimb lengths (TFL, HLL) are only significantly larger in males of *A. cisternasii.* Such differences could be the result of selection for improved manipulation and transport of eggs, although the trend may not be as clear in *A. obstetricans* because, on average, males of this species transport egg masses which are proportionally lower in weight in relation to male body mass (R. Márquez, unpublished data).

The discriminant functions obtained from our data allow for the determination of sexes based on a reasonable number of morphological variables in each species, at least for adult individuals of the populations studied. Actually, the percentage of correct classifications for a single variable (80% in *A. obstetricans* and 83% in *A. cisternasii,* may also be sufficient for some studies. Caution should be used in extending the results to individuals from populations of different taxonomic status or with samples that include sizes beyond the range used in our study. Arntzen & García París (1995) demonstrated a marked level of geographical variation within and between *Alytes* species, but this study did not consider sexual dimorphism within populations and some of the differences observed may well be attributable to differences in sample sex ratios between populations or species. We present our study as a methodological tool that could be of use in such instances, but data from individuals of known sex from the specific populations studied have to be used to generate each discriminant function. We suggest that the methodology used in our paper may be applied in studies of other populations of *Alytes* or other anurans with little sexual dimorphism, where determination of the sex ratio is a central aim. All measurements should be taken from an increasing number of individuals until enough individuals of confirmed sex are measured and a desired percentage of correct classification is reached. Then the discriminant functions may be obtained and the sex of the undetermined individuals observed previously can be determined *a posteriori*. Thereafter, only the variables included in the discriminant function should be measured on any new observed individuals of undetermined sex. Such a technique can be of use for the study of relict populations of *Alytes* such as the Mallorcan midwife toad, *A. muletensis,* whose recovery plan is currently being undertaken, and possibly to other threatened species of midwife toads, such as the newly-described *A. dickhilleni* (Arntzen & García-París, 1995), with highly isolated and reduced populations (Márquez, García-París & Tejedo, 1994). Similarly, the procedure can be of use for year-round field studies of populations of other species of anurans that may present secondary sexual characters only during the breeding season, such as species in the genera *Discoglossus* and *Rana.*

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