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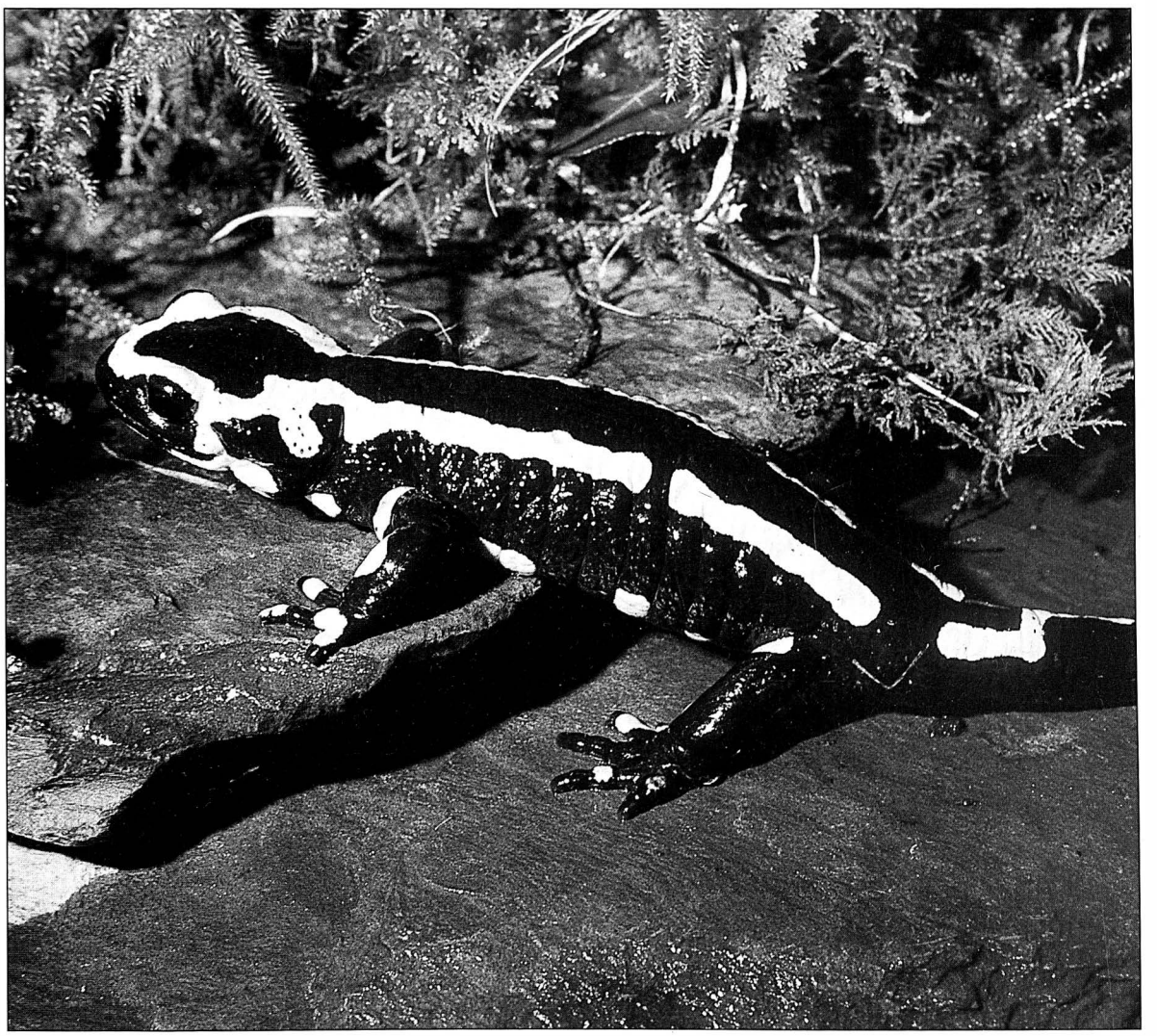

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# HYPERBILIVERDINEMIA IN THE SHINGLEBACK LIZARD (*TILIQUA RUGOSA*)

MARCELLO PENNACCHIO[1], MICHAELA BOGYI[1], LIKHIM TEH[1] AND EMILIO L. GHISALBERTI[2]

[1]*Department of Environmental Biology, Curtin University of Technology, GPO Box U1987, Perth, WA 6845, Australia*

[2]*Department of Chemistry, The University of Western Australia, Nedlands, WA 6907, Australia*

Green pigmentation in the serum of shingleback lizards (*Tiliqua rugosa*) was the result of an excess of the bile pigment biliverdin (hyperbiliverdinemia). This was confirmed by comparing the absorbance spectrum of the affected serum with that of commercial biliverdin, using TLC and acidification with both nitric and sulphuric acid. The average content of biliverdin in animals with hyperbiliverdinemia was 2.52±0.15 mg/100 ml. Significant changes in the packed cell volume, haemoglobin content, blood glucose levels, body mass and levels of erythropoietin were also observed in animals with this form of green jaundice. Interestingly, significant erythrocyte degeneration, especially in the stroma area of the red blood cells, appears to result in a significant release of haemoglobin into the blood serum, which may account for the excess levels of biliverdin. Changes in the haematology of shingleback lizards are discussed along with the probable cause for hyperbiliverdinemia.

*Key words*: bile pigments, biliverdin, jaundice, shingleback lizards

## INTRODUCTION

Green blood pigmentation has been reported for a variety of animals, including several species of fishes (see Fang & Bada, 1990 for a review); butterflies, moths (Kayser, 1985) and other insects (Law & Wells, 1989); frog eggs (Marinetti & Bagnara, 1983); lizards (Greer & Raizes, 1969), including skinks of the genus *Prasinohaema* of Papua New Guinea (Austin & Jessing, 1994); bird egg shell (Fox, 1976); dog placenta (Fox, 1976) and humans (Greenberg *et al.*, 1971). In all of these animals, the green colour was due to an excess of the bile pigment, biliverdin (hyperbiliverdinemia).

Biliverdin is a bilatriene compound produced during the metabolism of the haeme portion of haemoglobin (Britton, 1983). In most higher vertebrates, this transitory intermediate metabolite is rapidly oxidized into the more toxic bilirubin (Cowger, 1974). Reptiles, amphibians and birds lack the enzyme required for this process (biliverdin reductase) and therefore do not produce the latter compound. An increase in either of these bile pigments results in the pathological condition known as jaundice in most vertebrates.

While collecting blood from shingleback lizards (*Tiliqua rugosa*, Family Scincidae) for another study, it was noticed that the serum, normally pale yellow in colour, was green (Pennacchio, 2001). In this paper, we identify the pigment responsible for the abnormal coloration in the serum of *T. rugosa* (also referred to as *Trachydosaurus rugosus,* Cogger, 2000) and offer some explanation for the excess levels seen.

*Correspondence*: M. Pennacchio, Department of Environmental Biology, Curtin University of Technology, GPO Box U1987, Perth WA 6845. *E-mail:* M.Pennacchio@exchange.curtin.edu.au

## MATERIALS AND METHODS

### ANIMALS

Seven shingleback lizards, from the field trial area (FTA) at Curtin University of Technology's Department of Environmental Biology, were trapped using baited Sheffield traps and were housed together with shingleback lizards that once had hyperbiliverdinemia. A preliminary study had revealed that shingleback lizards kept with those previously affected by the condition also developed it. A permit to capture, collect and keep shingleback lizards was approved by the Department of Conservation and Land Management (CALM permit No. SF003566). The animals were fed and watered *ad libitum* and were maintained in an outdoor enclosure where they had access to sunlight.

### BLOOD EXTRACTION

Blood (0.2 ml) from shingleback lizards was obtained directly from the ventricle of the heart (heart puncture). This method was approved by Curtin University of Technology's Animal Experimentation and Ethics Committee (Approval No. N12/2001) and has been used extensively and successfully elsewhere without harming the animals.

Once collected, the blood was immediately transferred to sterile, heparinized Vacutainer® vials and was subsequently divided into smaller samples. A small volume was centrifuged in a Hawksley micro-haematocrit centrifuge to determine the packed cell volume (% red blood cells and % white blood cells). The haemoglobin content in whole blood and serum was determined using two separate HemoCue haemoglobin analysers, (blood haemoglobin and plasma/low) with HemoCue self-filling micro-cuvettes. Blood glucose levels were measured using a MediSense glucose analyser while levels of erythropoietin (EPO) were measured with a commer-

cially-available kit (EPO.96) obtained through MD Biosciences. Blood smears were air-dried and stained using a commercially available kit (Harlequin DiffQuick). Photographs of relevant blood smears, to be used in determing the percentage of RBC as well as the extent and nature of the damage to them, were taken using an Olympus Vanox microscope (model AHBS-513). The degeneration of erythrocytes was compared to similar damage reported in De V. Pienaar (1962). The remainder of the blood was centrifuged at 3000 RPM for five minutes to separate the formed elements from the serum. The serum was retained for spectrophotometric analyses used to detect the presence of biliverdin.

### SPECTROPHOTOMETRIC ANALYSES

The presence of biliverdin in serum was confirmed using four chemical tests (Fang, 1982). The first test involved the spectrophotometric analyses and comparison of green serum with normal pale yellow serum and pure biliverdin purchased from ICN Chemicals. Absorbance spectra, ranging from 350 nm to 800 nm, were measured using a Pharmacia Biochrom 4060 UV/Visible spectrophotometer. A standard curve, using commercial biliverdin, was prepared to determine the concentration of biliverdin in the green serum.

### THIN LAYER CHROMATOGRAPHY

Retardation factors ($R_f$) of pure biliverdin and blue-green serum were compared using thin layer chromatography (TLC). Small volumes of serum and commercial biliverdin were placed onto an aluminium TLC plate coated with silica gel (Merck 60 $F_{254}$) and prewashed with methanol to remove water. Each TLC plate was then partially immersed upright in a chamber with a 2:1:1.5 butanol:methanol:water mixture until sufficient separation had occurred.

### ACIDIFICATION

The third and fourth tests involved acidification of serum with concentrated nitric acid and sulphuric acid, respectively. In the first of these two tests, the Gmelin reaction, plasma proteins were removed from the serum by precipitation with ammonium sulphate (55 % w/v). This mixture was centrifuged at 3000 rpm for five minutes, after which the supernatant was recovered. Concentrated nitric acid was slowly added to the supernatant and observed for any visible changes in colour.

A small volume of concentrated sulphuric acid was then added to a separate sample of serum, which was gently heated. This process destroys biliverdin, but does not affect its isomer, mesobiliverdin (Fang, 1982).

### DATA ANALYSIS

Paired *t*-tests were performed on the data to determine differences in means between the shingleback lizards with and without hyperbiliverdinemia, using SPSS (v. 10.0). All data in the form of percentages were arcsine transformed prior to comparing means to ensure normal distribution (Zar, 1984). All results are presented as means ± SE. The number of animals used was seven unless otherwise stated.

## RESULTS

The green pigmentation in the serum of all seven of the shingleback lizards was the result of an excess accumulation of the bile pigment, biliverdin. A comparison of the absorbance spectra of sera with green pigmentation and with commercially available biliverdin revealed that the spectra were almost identical. Both sample types resulted in two bands, one of which occurred in the 380 and 450 nm range (normal for shingleback lizards), and a broader band at the 640-665 nm range (Fig 1). Normal pale- yellow shingleback- lizard serum lacks the broader band, which is characteristic of biliverdin (Fig. 1).

The absorbance spectra of sera with the green pigmentation were then compared to absorbances in the standard curve to determine the concentration of the bile pigment in each blue-green sample. The average concentration was 2.5±0.2 mg/100 ml during severe episodes of hyperbiliverdinemia (Table 1). The presence of biliverdin was also revealed by TLC. The $R_f$ of the green sera and that of the pure biliverdin were identical. All plates were left overnight to allow for adequate separation.

The Gmelin reaction revealed that the supernatant of sera treated with ammonium sulphate immediately changed colour from green to yellow upon acidification with concentrated nitric acid. This also suggested that the green pigment in the supernatant was in fact biliverdin. Acidification of green serum with concentrated sulphuric acid resulted in complete discoloration, indicating that the pigment was indeed biliverdin and not its isomer mesobiliverdin.

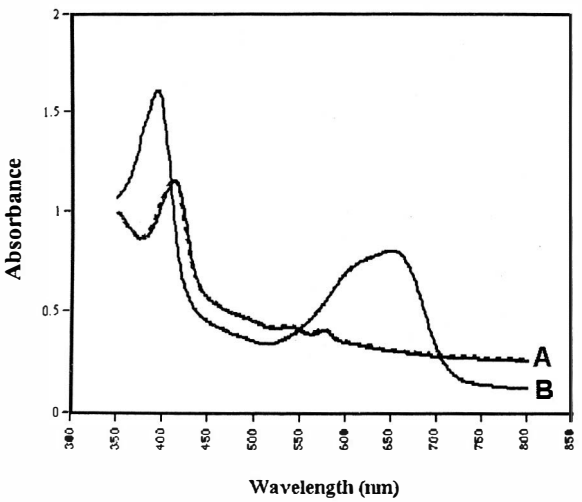


FIG. 1 Absorbance spectra of normal shingleback lizard serum (A) and green-pigmented serum with hyperbiliverdinemia (B)

TABLE 1. Changes in the haematology and other parameters of shingleback lizard with and without hyperbiliverdinemia. Key to table: Hyper, hyperbiliverdinemia; Damage, percentage of damage to erythrocytes seen in blood smears; RBC, red blood cells (erythrocytes); WBC, white blood cells (leucocytes); Hb, haemoglobin content of whole blood; LHb, low haemoglobin content of serum; EPO, erythropoietin.

| | Damage (% RBC) | Biliverdin (mg/100 ml) | RBC (%) | WBC (%) | Hb (g/dL) | LHb (g/L) | EPO (mU/L) | Glucose (mmol/L) | Body Mass (g) |
|---|---|---|---|---|---|---|---|---|---|
| Normal | 0.0±0.0 | 0.0±0.0 | 18.6±1.4 | 1.0±0.3 | 7.8±4.5 ($n$=4) | 0.0±0.0 | 1.4±2.2 ($n$=4) | 7.9±2.4 | 378.1±126.0 |
| Hyper | 31.8±11.2 | 2.52±0.2 | 11.3±1.6 | 3.1±0.9 | 3.9±1.5 ($n$=4) | 1.8±0.6 | 32.3±0.0 ($n$=4) | 5.7±1.7 | 327.7±103.6 |

The packed cell volumes of both the normal and green-pigmented blood were also compared (Table 1). There was a significant difference ($P$=0.000) in the percentages of white blood cells (WBC; leucocytes) between the two groups. Up to five times as many WBC were observed in the blood of some affected animals. The average percentage of leucocytes was, however, 1.0±0.3% at the start and 3.1±0.9% during bouts of severe hyperbiliverdinemia. Erythrocytes, which averaged 18.6±1.4% in normal animals, decreased significantly ($P$=0.003; Table 1) to 11.3±1.6% in animals with hyperbiliverdinemia. Interestingly, there was a significant ($P$=0.008) increase in the RBC percentage during the first two weeks, when it increased to 24.2±1.7%. This occurred prior to the animals developing hyperbiliverdinemia.

Shingleback lizards with hyperbiliverdinemia also exhibited a significant ($P$=0.002) degeneration of erythrocytes (Table 1). An average of 31.8±11.2% of all erythrocytes seen in blood smears derived from affected animals was associated with intraerythrocytic inclusions (albuminoid vacuoles). These included extensive aniso- and poikilo-cytosis, as well as cellular distortion and cytolysis of the stroma. Coinciding with the destruction of erythrocytes were significant increases in biliverdin levels ($P$=0.001), as well as significant decreases in haemoglobin content in the sera ($P$=0.003; Table 1) and EPO levels ($P$=0.019; Table 1). The haemoglobin content in whole blood decreased significantly ($P$=0.016; Table 1). A number of phagocytic lymphocytoid azurophils (WBC), which had ingested damaged erythrocytes, were clearly visible in some blood smears.

Finally, there was a significant decrease in both body mass and blood glucose levels of shingleback lizards with excess biliverdin. These decreased from 378±126.0 g to 327.7±103.6 g ($P$=0.034) for body mass and 7.9±2.4 mmol/L to 5.7±1.7 mmol/L for blood glucose levels ($P$=0.002). Most shingleback lizards presented normal haematology after developing hyperbiliverdinemia, but it was not clear precisely how long the condition lasted and why it soon recurred in some of our animals. This is currently the focus of another study.

## DISCUSSION

All four of our tests confirmed that the distinct green serum of the seven shingleback lizards was in fact due to excess biliverdin. Researchers have proposed a number of hypotheses to account for green jaundice in animals. Yamaguchi & Hashimoto (1968), for example, reported that hyperbiliverdinemia appears to help with lipid transport in some species and may protect them from UV rays (Yamaguchi *et al.*, 1976). Low & Bada (1974) reported that the condition assists with cryptic coloration in some animals, as it may manifest itself in the green coloration of their exteriors. Schwalm *et al.* (1977) and Emmerson *et al.*, (1990), in contrast, have suggested that the green serum appears to confer advantages to thermoregulation for animals with excess

biliverdin. High levels of biliverdin may even make the animals "distasteful" to their predators (Austin & Jessing, 1994). Most of these hypotheses have not been seriously tested.

In contrast, hyperbiliverdinemia in shingleback lizards appears to be the result of erythrocyte degeneration (erythrolytic jaundice), resulting in a significant increase in the content of haemoglobin in the serum of affected animals. Similar findings were reported by Maeno *et al.* (1995) who recently suggested that erythrocyte destruction was responsible for increases in serum haemoglobin and bilirubin concentrations in jaundiced yellowtail fish (*Seriola quinueradiata*). Maeno *et al.* (1995) suggested that a bacterium was the likely causative agent.

The increase in WBC seen during episodes of shingleback-lizard hyperbiliverdinemia suggests that an immune response had taken place and that possibly a contagious parasite may be responsible for the destruction seen in their erythrocytes. This may also be inferred from the fact that the animals developed the condition upon contact with previously affected animals.

At first, Pirhemocyton-like infection of erythrocytes by viruses was suspected. This had previously been reported for a number of Australian lizards (Paperna & Alves de Matos, 1993) and other reptiles (Daly *et al.*, 1980; Alves de Matos & Paperna, 1993; Telford & Jacobson, 1993), as well as for frogs (Alves de Matos *et al.*, 1995) and ornamental fish (Paperna *et al.*, 2001). It was not clear what type of pathogen was responsible for the degeneration of shingleback- lizard erythrocytes.

The elevated EPO levels observed during the development of hyperbiliverdinemia may also be due to erythrocyte degeneration. It was not clear at this stage, but it is thought that EPO plays a role in the haematopoietic recovery of shingleback lizards with excess biliverdin levels. The kit used in these experiments was, however, for human EPO and therefore provides only limited evidence that the serum of shingleback lizards with hyperbiliverdinemia contains an immunoreactive erythropoietin-like molecule that,with an increase in damage to erythrocytes, results in the higher levels. A similar kit was used by Wickramasinghe *et al.* (1994) to provide evidence that teleost kidneys are erythropoietic-producing organs.

The significant decreases in body mass and blood-glucose levels are interesting but cannot be explained based on the data gathered in this study. The intake of food and blood glucose level decreases in shingleback lizards affected by hyperbiliverdinemia is currently the subject of a more detailed study into the long-term effects of the condition on the animals. The study also aims to determine the time it takes for animals to recover from the condition and why some later relapse. It is hoped that studies of this type will help with our understanding of hyperbiliverdinemia in shingleback lizards and with the pathology of jaundice in humans and other animals (Colleran & O'Carra, 1977; Fang & Bada, 1990).

## ACKNOWLEDGMENTS

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## REFERENCES

Alves De Matos, A. P. & Paperna, I. (1993). Ultrastructural study of Pirhemocyton virus in lizard erythrocytes. *Annales de Parasitologie Humaine et Comparée* **68**, 24-33.

Alves De Matos, A. P. , Paperna, I. & Lainson, R. (1995). An erythrocytic virus of the Brazilian tree-frog, *Phrynohyas venulosa. Memórias do Instituto Oswaldo Cruz.* **90**, 653-655.

Austin, C. C. & Jessing, K. W. (1994). Green-blood pigmentation in lizards. *Comparative Biochemistry and Physiology* **109A**, 619-626.

Britton, G. (1983). *The biochemistry of natural pigments.* Cambridge University Press, UK.

Cogger, H. G. (2000). *Reptiles and amphibians of Australia.* 6th ed. Reed New Holland Publishers, Sydney.

Colleran, E. & O'Carra, P. (1977). Enzymology and comparative physiology of biliverdin reduction. In Berck, P.D. and Berlin, N.I. (Eds.). *Chemistry and physiology of bile pigments.* Bethesda. National Institute of Health.

Cowger, M. L. (1974). Toxicity and protein binding of biliverdin and other bile pigments. In Odell *et al. Phytotherapy in the newborn: an overview.* National Academy of Sciences, Washington.

Daly, J. J, Mayhue, M., Menna, J. H. & Calhoun, C. H. (1980). Virus like particles associated with Pirhemocyton inclusion bodies in the erythrocytes of a water snake, *Nerodia erytrogaster flavigaster. Journal of Parasitology* **66**, 82-87.

De V. Pienaar, U. (1962). *Haematology of some South African reptiles.* Witwatersrand University Press, South Africa.

Emmerson, S. B., Cooper, T. A. & Ehlinger, J. R. (1990). Convergence in reflectance spectra among treefrogs. *Functional Ecology* **4**, 47-51.

Fang, L. S. (1982). *The blue-green pigment in the blood serum of a marine fish,* Clinocottus analis*: identification, metabolism and biological significance.* Ph.D Thesis, University of California, San Diego, CA.

Fang, L. S. & Bada, J. L. (1990). The blue-green blood plasma of marine fish. *Comparative Biochemistry and Physiology* **97B**, 37-45.

Fox, D. L. (1976). *Animal biochromes and structural colours*. University of California Press, Berkeley, CA.

Greenberg, A. J., Bossenmaier, I., Schwartz, B. A. & Schwartz, S. (1971). Green jaundice. *American Journal of Digestive Diseases* **16**, 873-880.

Greer, A. E. & Raizes, G. (1969). Green blood pigment in lizards. *Science* **166**, 392-393.

Kayser, H. (1985). *Comprehensive insect physiology, biochemistry and pharmacology*. Vol. 10. Pergamon Press, Oxford.

Law, J. H. & Wells, M. A. (1989). Insects as biochemical models. *Journal of Biological Chemistry* **264**, 16355 - 16338.

Low, P. S. & Bada, J. (1974). Bile pigments in the blood serum of fish from the family Cottidae. *Comparative Biochemistry and Physiology* **47A**, 411-418.

Maeno, Y., Nakajima, K., Sorimachi, M. & Inui,Y. (1995). Pathophysiological studies of jaundiced yellowtail, *Seriola quinqueradiata*. *Fish Pathology* **30**, 7-14.

Marinetti, G. V. & Bagnara, J. T. (1983). Yolk pigments of the Mexican leaf frog. *Science* **219**, 985- 987.

Paperna, I. & Alves de Matos, A. P. (1993). Erythrocytic viral infections of lizards and frogs: new hosts, geographical locations and description of the infection process. *Annales de Parasitologie Humaine et Comparée* **68**, 11-23

Paperna, I., Vilenkin, M. & Alves de Matos, A. P. (2001). Iridovirus infections in farm-reared tropical ornamental fish. *Diseases of Aquatic Organisms* **48**, 17-25.

Pennacchio, M. (2001) Green-blooded lizards. *Wildlife Australia Magazine* **38**, 14.

Schwalm, P. A., Starrett, P. H. & McDiarmid, R. W. (1977). Infrared reflectance in leaf-splitting neotropical frogs. *Science* **196**, 1225-1227.

Telford, Jr. S. R. & Jacobson, E. R. (1993). Lizard erythrocytic virus in east African chameleons. *Journal of Wildlife Diseases* **29**, 57-63.

Wickramasinghe, S. N., Shiels, S. & Wickramasinghe, P. S. (1994). Immunoreactive erythropoietin in teleosts, amphibians, reptiles and birds. Evidence that the teleost kidney is both an erythropoietic and erythropoietin-producing organ. *Annals of the New York Academy of Sciences* **718**, 366-370.

Yamaguchi, K. & Hashimoto, K. (1968). Studies on a blue-green serum pigment of eel III. Amino acid composition and constituent sugars. *Bulletin of the Japanese Society of Scientific Fisheries* **34**, 214-219.

Yamaguchi, K., Hashimoto,K. & Matsuura, F. (1976). Identity of blue pigments obtained from different tissue of the sculpin, *Pseudoblennius percoides* Gunther. *Comparative Biochemistry and Physiology* **55B**, 85 – 87

Zar, J. H. (1984). *Biostatistical analysis*. 2nd ed. Prentice-Hall International editions, New Jersey.

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# EFFECT OF INTRODUCED FISH ON AMPHIBIAN SPECIES RICHNESS AND DENSITIES AT A MONTANE ASSEMBLAGE IN THE SIERRA DE NEILA, SPAIN

I. MARTÍNEZ-SOLANO[1], L. J. BARBADILLO[2] AND M. LAPEÑA[2]

[1]*Museo Nacional de Ciencias Naturales, CSIC, Madrid, Spain*

[2]*Departamento de Biología, Unidad de Paleontología, Universidad Autónoma de Madrid, Madrid, Spain*

We examined the effect of fish stocking practices on the populations of seven amphibian species in a montane area in the Sierra de Neila (north-central Spain). We compared values for amphibian species richness and amphibian densities between ponds where fish have been introduced and ponds where fish are absent. Our results show that (1) amphibian species richness was significantly lower in ponds where fish have been introduced; (2) we found contrasting patterns of pond occupancy by the different amphibian species: on the one hand, two out of seven species (*Bufo bufo* and *Rana perezi*) coexist with fish, whereas the other five species breed exclusively in ponds where fish are absent; (3) based on comparisons of presence/ absence data for species present in the area in 1981, 1991 and 2001, we concluded that two amphibian species have suffered severe declines in the last decades. Presently, *Alytes obstetricans* is almost exclusively confined to a few fishless streams, whereas *Salamandra salamandra* appears to have been completely extirpated from the whole area. This local decline of *S. salamandra* seems to be general for the whole region of the Sistema Ibérico (North-central Spain). The possible role of fish stocking practices in these declines is discussed.

*Key words*: amphibian decline, conservation, exotic fish, Spain

## INTRODUCTION

Multiple causes have been proposed to explain documented patterns of amphibian declines throughout the world (reviewed, for example, in Alford & Richards, 1999). Among these factors, much interest has focused on analysing the effect of exotic fish on amphibian populations. Several cases of exclusion – and even extinction (Bradford, 1991) – of amphibian species from ponds after the introduction of non-native fish species have been reported in the literature (Aronsson & Stenson, 1995; Drost & Fellers, 1996; Fisher & Shaffer, 1996; Gamradt & Kats, 1996; Galán, 1997). Allotopic patterns of distribution of introduced fish and amphibians, as well as reduced values of amphibian species diversity in ponds stocked with fish, are both well documented at local (Woodward, 1983; Bradford, 1989; Brönmark & Edenham, 1994; Braña *et al.*, 1996) and regional scales (Hecnar & M'Closkey, 1997).

We have explored the effect of fish stocking practices during recent decades on amphibian species richness and densities in a montane protected area in the Sierra de Neila, in Burgos (north-central Spain). The zone is appropriate for the study of the effect of fish on amphibian populations because of the coexistence of ponds where several species of fish have been introduced and ponds where fish are absent. There is evidence that the populations of some of the amphibian species present at the area (for example, *Salamandra salamandra*) have strongly declined over recent decades, a trend that might also be occurring at a higher (regional) scale (Barbadillo & García-París, 1991; Lizana & Barbadillo, 1997; Barbadillo & Sánchez-Herráiz, 1998; Barbadillo, unpublished data). The availability of amphibian presence/absence data for several water bodies in the study area in the years 1981 and 1991 led us to conduct a more comprehensive field study in 2001. Our objectives were (1) to document possible changes in amphibian species composition in recent years by analysis of data collected over the last two decades; and (2) to explore the effects of introduced fish on the relative abundance of the amphibian species breeding in the study area.

*Correspondence*: I. Martínez-Solano, Museo Nacional de Ciencias Naturales, CSIC. José Gutiérrez Abascal, 2. 28006 Madrid, Spain. *E-mail*: mcnim548@mncn.csic.es

## MATERIALS AND METHODS

The study area is located in the Sierra de Neila and included within the limits of the protected area of the "Espacio Natural de la Sierra de la Demanda" (81270 ha), in SE Burgos (Sistema Ibérico, north-central Spain). The area consists mainly of alpine grasslands surrounded by *Pinus sylvestris* forests. The ponds studied in 2001 are of glacial origin and include Laguna Negra, Laguna de la Cascada, Laguna de los Patos, Laguna Brava, Laguna de las Pardillas, Laguna Haedillo and Laguna Oruga (Fig. 1). We also sampled two temporary ponds located in the vicinities of the Laguna Cascada. At Laguna Haedillo we only recorded presence or absence of amphibian species because of problems in carrying out nocturnal transects posed by difficult access in relation to the other ponds. In addition, other sites where historical records of *S. salamandra* were available (Fuente Sanza and Fuente Rialares) were sampled in order to determine their presence or absence in 2001.

According to data from the "Consejería de Medio Ambiente" of the "Junta de Castilla y León", between 1976 and 1995 three of the studied ponds (Negra, Patos

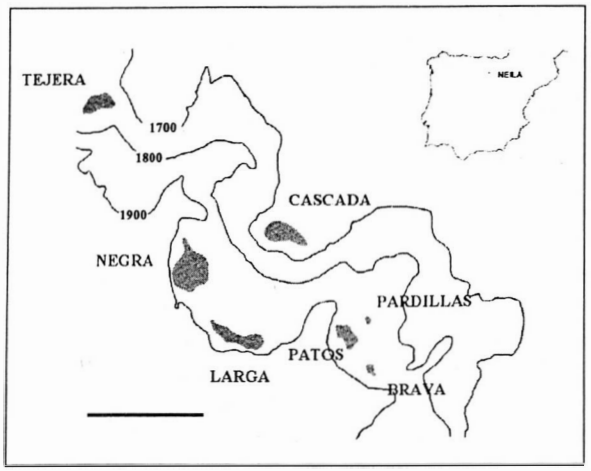


FIG 1. Study area. Location of Neila in the Iberian Peninsula (upper-right corner) and ponds that were sampled in 2001 (except for Laguna Oruga, which is located 8 km east of Laguna Patos; and Laguna Haedillo, located 4 km north-west of Laguna Negra). Scale bar equals 1 km.

and Cascada) were used as sport fishing reserves (no data were available for Laguna Brava). These ponds were stocked with brown trout (*Salmo trutta*), rainbow trout (*Oncorhynchus mykiss*) or both species. During those years, 69 000 kg (4000 kg/yr on average) of these fish species were released into the ponds. Apart from these official stocking practices, uncontrolled introductions of cyprinid species have also taken place: *Cyprinus carpio, Gobio gobio*, *Phoxinus phoxinus*, *Carassius auratus* and *Chondrostoma arcasii* have been identified in one or several of the ponds (C. Temiño, Junta de Castilla y León, personal communication; authors, personal observations).

Eight amphibian species have been reported to breed in the area: *Salamandra salamandra, Triturus helveticus*, *T. marmoratus*, *Alytes obstetricans*, *Bufo bufo*, *B. calamita*, *Hyla arborea*, and *Rana perezi* (Barbadillo & Sánchez-Herráiz, 1998; Barbadillo, unpublished data, Table 1).

We visited the area weekly during the months of May to July 2001, coinciding with the period of reproductive activity of amphibians at the area (Barbadillo, 1987), in order to obtain estimates of adult amphibian densities at each pond. Densities for each species were obtained from standardized transects that included visual encounters – both during the day and at night – and nocturnal acoustic transects (see Heyer *et al.*, 1994). We dip-netted some of the ponds in order to detect amphibian larvae and thus confirm the presence of breeding populations at each pond. We also carried out standardized transects of fixed length in which we visually recorded fish densities. Electrofishing or fishing nets were discarded because of difficulties imposed by the large extent, depth, and rocky bottom of most of the ponds. We scored separately presence/absence and densities of salmonids and cyprinids. Densities of amphibians and fish were scored as number of specimens per metre of transect. Maximum, minimum and average density values across visits were calculated. The small, peripheral ponds located in the vicinities of Laguna Cascada dried very early in the season and data about amphibian densities were only available for one of the visits.

In order to document possible changes in species richness at each pond, we compared presence/absence data for each amphibian species at each pond between previous, non-standardized surveys carried out in 1981 and 1991 and the results of 2001 samplings. In the samplings of 1981 and 1991 we obtained data on the amphibian species breeding at each pond. These samplings (1981 and 1991) also included multiple visits during the breeding season which combined nocturnal transects in order to detect adults and diurnal transects and dip-netting in the ponds selected in order to detect larvae of the different species. In all cases, presence was scored for the analyses only when evidence of reproductive activity was detected (presence of calling males, clutches or larvae).

We also compared present values of amphibian species richness and densities between fish-stocked ponds and fishless ponds. Again, only data for species that breed at each of the ponds were considered. We tested for correlation of fish and amphibian densities, considering separately each of the seven amphibian species breeding at the area. For the statistical analyses, only maximum densities were used as they were assumed to reflect more precisely the potential of a pond to hold amphibian populations while being at the same time less sensitive to sampling bias.

To explore the relationships between the presence/absence and the densities of each of the amphibian species and the characteristics of the ponds, we recorded the following variables: pond area, pond depth, percentage of aquatic vegetation on the surface, and type of substrate at the bottom of each pond. This latter variable was calculated as the ratio between the percentage of the bottom of the pond composed by rocks and the percentage of the bottom composed by mud. Because most ponds were quite large and the visibility of the bottom was restricted to a few meters form the shore, the percentages refer to the characteristics of the bottom of the pond in a two-meter wide ring immediately adjacent to the shore all along the perimeter of the pond. All comparisons were made using non-parametric statistical analyses (Spearman correlations).

## RESULTS

Salmonids and cyprinids were both present in Lagunas Brava, Negra, and Patos (Table 1). The maximum densities of cyprinids were observed at Lagunas Negra and Patos (2.250 and 2.010 individuals per metre of transect, respectively). Only Laguna Cascada contained cyprinids (in low densities, only 0.005 individuals/metre) but not salmonids. As commented above, this pond was stocked with salmonids in recent decades, but in our surveys in 2001 we only found cyprinids. The present lack of salmonids in this pond was probably related to

TABLE 1. Presence (+) or absence (-) of the eight amphibian species at Neila in ponds sampled in 1981, 1991 and 2001. For amphibians, presence was scored only when evidence of reproduction was recorded. For *A. obstetricans*, +(-) indicates that, although calling males were detected in the surroundings of the ponds, no larvae were detected during the surveys at those sites.

| | *S. salamandra* | | | *T. helveticus* | | | *T. marmoratus* | | | *A. obstetricans* | | | *B. bufo* | | | *B. calamita* | | | *H. arborea* | | | *R. perezi* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1981 | 1991 | 2001 | 1981 | 1991 | 2001 | 1981 | 1991 | 2001 | 1981 | 1991 | 2001 | 1981 | 1991 | 2001 | 1981 | 1991 | 2001 | 1981 | 1991 | 2001 | 1981 | 1991 | 2001 |
| WITH FISH | | | | | | | | | | | | | | | | | | | | | | | | |
| Brava | | | - | | | - | | | - | | | +(-) | | | + | | | - | | | - | | | + |
| Negra | + | - | - | + | - | - | - | - | - | + | + | +(-) | + | + | + | - | - | - | - | - | - | + | + | + |
| Patos | | | - | | | - | | | - | | | +(-) | | | + | | | - | | | - | | | + |
| Cascada | | | - | | | + | | | + | | | + | | | + | | | - | | | + | | | + |
| FISHLESS | | | | | | | | | | | | | | | | | | | | | | | | |
| Casc. (per. 1) | | | - | | | + | | | + | | | - | | | - | | | + | | | + | | | + |
| Casc. (per. 2) | | | - | | | + | | | + | | | - | | | - | | | + | | | + | | | + |
| Haedillo | + | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Oruga | + | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | + | + | + | + | + |
| Pardillas | + | - | - | + | + | + | + | + | + | + | + | +(-) | + | + | + | + | + | - | + | + | + | + | + | + |
| Fuente Sanza | + | + | - | | | | | | | | | | | | | | | | | | | | | |
| Fuente Ria. | + | + | - | | | | | | | | | | | | | | | | | | | | | |

TABLE 2. Maximum value ($D_{max}$), average (Av) and standard deviation (SD) of the densities recorded at each pond for the amphibian species present at the study area. Average densities were calculated from the values obtained at each visit to a certain pond; for ponds with fish and those peripheral ponds only one record was available.

| | Salm. | Cypr. | *T. helveticus* | | | *T. marmoratus* | | | *A. obstetricans* | | | *B. bufo* | | | *B. calamita* | | | *H. arborea* | | | *R. perezi* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | $D_{max}$ | Av | SD | $D_{max}$ | Av | SD | $D_{max}$ | Av | SD | $D_{max}$ | Av | SD | $D_{max}$ | Av | SD | $D_{max}$ | Av | SD | $D_{max}$ | Av | SD |
| FISH | | | | | | | | | | | | | | | | | | | | | | | |
| Brava | 0.020 | 0.180 | | | | | | | | | | 1.292 | 0.435 | 0.554 | | | | | | | 0.041 | 0.017 | 0.018 |
| Negra | 0.024 | 2.250 | | | | | | | | | | 1.418 | 0.449 | 0.582 | | | | | | | 0.110 | 0.044 | 0.044 |
| Patos | 0.015 | 2.010 | | | | | | | | | | 0.340 | 0.121 | 0.126 | | | | | | | 0.140 | 0.056 | 0.060 |
| Cascada | | 0.005 | 0.076 | 0.043 | 0.029 | 0.005 | 0.002 | 0.002 | 0.014 | 0.006 | 0.006 | 0.426 | 0.169 | 0.185 | | | | 0.014 | 0.005 | 0.007 | 0.143 | 0.053 | 0.064 |
| FISHLESS | | | | | | | | | | | | | | | | | | | | | | | |
| Casc. (per. 1) | | | 0.513 | 0 | | 0.162 | 0 | | | | | | | | 0.135 | 0 | | 0.081 | 0 | | 0.027 | 0 | |
| Casc. (per. 2) | | | 0.411 | 0 | | 0.109 | 0 | | | | | | | | 0.109 | 0 | | 0.027 | 0 | | 0.027 | 0 | |
| Pardillas | | | 0.037 | 0.012 | 0.011 | 0.061 | 0.027 | 0.024 | | | | 0.035 | 0.013 | 0.011 | | | | 0.207 | 0.038 | 0.065 | 0.700 | 0.232 | 0.235 |

TABLE 3. Relationships between pond characteristics, density of predatory fish, and maximum densities of amphibian species at Neila (results of Spearman non-parametric tests). For densities of *R. perezi*, only data on their relationship with fish densities was included because this species breeds in all the ponds studied. *A. obstetricans* was not included because we found evidence of reproduction of this species in only one of the ponds studied. N.S., $P>0.05$.

| Variable | | *Triturus marmoratus* | *Triturus helveticus* | *Bufo bufo* | *Bufo calamita* | *Hyla arborea* | *Rana perezi* | Salmonids | Cyprinids |
|---|---|---|---|---|---|---|---|---|---|
| *Area* | | | | | | | | | |
| | $r_s$ | -0.78 | - | 0.88 | - | | - | - | 0.86 |
| | $t$ | -2.77 | - | 4.20 | - | - | | - | 4.17 |
| | $P$ | 0.039 | N.S. | 0.008 | N.S. | N.S. | | N.S. | 0.006 |
| *Submerged veg.* | | | | | | | | | |
| | $r_s$ | 0.96 | 0.92 | -0.93 | 0.79 | 0.90 | | -0.87 | -0.88 |
| | $t$ | 7.09 | 5.11 | -5.54 | 2.85 | 4.55 | | -4.25 | -4.63 |
| | $P$ | <0.001 | 0.004 | 0.003 | 0.036 | 0.006 | | 0.005 | 0.003 |
| *Substrate* | | | | | | | | | |
| | $r_s$ | -0.92 | -0.85 | 0.79 | - | -0.92 | | 0.84 | 0.89 |
| | $t$ | -5.37 | -3.55 | 2.84 | - | -5.37 | | 3.84 | 4.83 |
| | $P$ | 0.003 | 0.016 | 0.036 | N.S. | 0.003 | | 0.009 | 0.003 |
| *Depth* | | | | | | | | | |
| | $r_s$ | -0.99 | -0.95 | 0.887 | -0.82 | -0.93 | | 0.84 | 0.92 |
| | $t$ | -15.97 | -6.92 | 4.30 | -3.17 | -5.75 | | 3.85 | 5.57 |
| | $P$ | <0.001 | <0.001 | 0.008 | 0.02 | 0.002 | | 0.008 | 0.001 |
| *Salmonids* | | | | | | | | | |
| | $r_s$ | -0.86 | -0.86 | 0.82 | - | -0.86 | -0.39 | 1.00 | - |
| | $t$ | -3.75 | -3.75 | 3.15 | - | -3.75 | -0.09 | - | - |
| | $P$ | 0.013 | 0.013 | 0.025 | N.S. | 0.013 | N.S. | - | - |
| *Cyprinids* | | | | | | | | | |
| | $r_s$ | -0.92 | -0.85 | 0.86 | - | -0.92 | 0.04 | 0.90 | 1.00 |
| | $t$ | -5.37 | -3.55 | 3.77 | - | -5.37 | 0.08 | 5.08 | - |
| | $P$ | 0.003 | 0.016 | 0.013 | N.S. | 0.003 | N.S. | 0.002 | - |

recent restoration practices that involved removal of artificial dams, which affected water level and probably made it an unsuitable habitat for these fish species. In fact, according to data from the "Consejería de Medio Ambiente", when this pond was emptied in 1999 very few salmonids (fewer than 10) remained (C. Temiño, pers. comm.).

Present values for amphibian species richness in the ponds studied in 2001 ranged from two to seven species (Table 1). The lowest values (two species of amphibian) were found at ponds that presently have fish (Lagunas Negra, Brava and Patos), whereas the highest values were found in fishless ponds (seven species of amphibian in Haedillo and Oruga, and five species in Pardillas and the peripheral ponds near Laguna Cascada). Six species of amphibian were breeding in 2001 at Laguna Cascada, where only cyprinids (but not salmonids) are present.

According to the comparisons of amphibian presence/absence data from the sampling carried out in 1981, 1991 and 2001, two out of eight species have experienced severe declines at the area: *S. salamandra* and *A. obstetricans*. In the case of *Salamandra salamandra*, we did not find adults or larvae in any of the sampled sites during the surveys in 2001, which indicates the extinction of all of the six populations that were known at the study area in 1981 (Table 1).

With respect to *Alytes obstetricans*, we detected calling males at very low densities (~10 calling males per night) in all of the ponds where salmonids were present (Negra, Brava and Patos). However, in 2001 surveys, no larvae were detected in any of these ponds. At Laguna Cascada, where cyprinids but not salmonids are present, we have detected larvae of *Alytes obstetricans*, but at very low densities compared with the density values obtained in the fishless pond Laguna Oruga (0.01 *vs.* 0.93 larvae per metre of transect, respectively).

There is a significant negative relationship between amphibian species numbers and presence of fish ($r$=-0.84, df=7, $P$=0.005) and also between amphibian species richness and density of fish (for salmonids: Spearman tests: $r_s$=-0.82; $n$=9, $P$=0.007; for cyprinids: $r_s$=-0.78, $n$=9, $P$=0.013). The relationship between amphibian and fish densities varied among species. In fact, in ponds where salmonids are present only *B. bufo* and *R. perezi* are abundant and breed successfully.

The highest values of densities of both species of *Triturus* were recorded at ponds where salmonids were absent (Table 2). The newts were, however, also present at Laguna Cascada (Table 1). The same pattern

(a significant negative correlation between maximum or average densities of amphibians and densities of salmonids) was evidenced for *H. arborea*. *B. calamita* was only detected in fishless, temporary ponds, although density values were always very low (Table 2).

On the other hand, *B. bufo* is relatively abundant in ponds that have been stocked with fish. The highest densities of *B. bufo* correspond to the ponds with higher densities of salmonids (1.29 and 1.41 adults of *B. bufo* per metre of transect at Lagunas Brava and Negra, Table 2). Consequently, the relationship between *B. bufo* and fish densities is positive and significant (Table 3).

The occupation by *R. perezi* of the ponds studied is apparently unaffected by the presence or absence of fish (Table 3). This species is present in all the ponds sampled (Table 1), although the highest densities were recorded at a fishless pond (0.70 individuals per metre at Laguna Pardillas, Table 2).

According to the results of the analyses of the relationship between pond characteristics and amphibian densities, the most important variables affecting the latter are the type of substrate, pond depth, and density of submerged vegetation (Table 3). Except for pond area, which was positively correlated with the density of *B. bufo* and negatively correlated with *T. marmoratus,* the remaining variables studied clustered amphibians and fish in two groups. *B. bufo* and salmonids are present in deep ponds with little submerged vegetation and rocky bottoms. On the other hand, *T. marmoratus, T. helveticus, B. calamita* and *H. arborea* breed only in shallow ponds with abundant submerged vegetation and muddy bottoms.

## DISCUSSION

The magnitude of the effect of exotic fish on amphibian populations is usually different among species. Not all amphibian larvae are equally vulnerable to predatory fish. For example, Hecnar & M'Closkey (1997) found that, among the species they studied, ranids and bufonids coexisted successfully more frequently with predatory fish than hylids or ambystomatids. We also found this pattern at Neila: only *B. bufo* and *R. perezi* breed successfully in ponds occupied by salmonids. Coexistence of amphibians and predatory fish is generally due to the existence of species-specific predator-avoidance mechanisms (Petranka *et al.*, 1987; Manteifel, 1995). Kats *et al.* (1988) stated that the existence of these mechanisms is best predicted by the frequency of encounter with fish rather than by phylogeny. According to this, amphibian species affected more severely by fish introductions would be those that have not been historically exposed to predatory fish: that is, species that breed in ephemeral ponds. This pattern is evident in our study area. *T. helveticus*, *T. marmoratus* and *H. arborea* are species that usually breed either in temporary ponds or in permanent, fishless ponds (Barbadillo *et al.*, 1999). In Neila they appear to be unable to coexist successfully with fish in permanent ponds, where marginal favourable breeding sites are present. In fact, in our study area, in accordance with other studies on breeding habitat selection, *T. marmoratus* and *H. arborea* selected ponds with abundant aquatic vegetation that provides oviposition sites for newts and support in the water for calling males (Miaud, 1995; Moravec, 1989). On the other hand, *B. bufo* was present in large, permanent ponds with scarce vegetation, a pattern that is general in other high altitude areas in Spain (personal observations). This pattern suggests that habitat requirements of *B. bufo* and fish overlap to a great extent in Neila.

Only two of the seven amphibian species breeding at Neila presently coexist with fish. In other mountainous areas in Central Spain, *B. bufo* usually shares reproduction sites with native salmonid species, such as the trout *Salmo trutta* (personal observations). The situation of long historical coexistence with *S. trutta* suggests that the impact of predation on *B. bufo* populations is probably low.

The case of *R. perezi* is different because they are present in almost every body of water in the study area, independently of the water body characteristics, including presence or absence of fish. *R. perezi* is a ubiquitous species that rapidly colonizes almost every body of water available, including large, permanent ponds (Llorente & Arano, 1997). Galán (1997) found that out of eight amphibian species, only *R. perezi* was able to breed successfully after the introduction of fish (*Gambusia affinis* and *Carassius auratus*) and crayfish (*Procambarus clarki*), although their densities were much lower than before introduction. To our knowledge, avoidance mechanisms or unpalatability in *R. perezi* larvae have not been described.

The remaining five species of amphibian are absent from ponds where fish have been introduced. Of these, three of them (*T. marmoratus, T. helveticus* and *H. arborea*) are locally abundant in fishless ponds. The remaining two species (*S. salamandra* and *A. obstetricans*) are respectively, absent or very scarce in the study area. *S. salamandra* has not been seen in the area since 1991. Although this species was not abundant at the Sierra de Neila (only six localities were known in the area in 1981, Table 1), the sampling effort in 2001 was higher than that of previous studies, and thus it is unlikely that they have been overlooked. Populations of this species located at lower altitudes in the same region of the study have also disappeared. This is the case in the two localities in the Sierra de Neila that still held salamanders in 1991, which were sampled in 2001 with negative results. The lack of evidence of reproduction of the species in the last decade suggests that *S. salamandra* populations at Neila might be very close to regional extinction. *S. salamandra* is likely to have been strongly affected by repeated fish introductions, although other causes (i.e. epidemic disease, such as chytridiomycosis) might be also involved. In other high altitude areas in Spain it has been shown to be extirpated from ponds and streams where fish were introduced (Martínez-Solano *et al.*, pers. obs.). An indirect effect of fish as vectors of

amphibian pathogens cannot be discarded as some cases of this interaction have already been reported (Kiesecker *et al.*, 2001). The species has also disappeared from other sites in the Sistema Ibérico in the province of La Rioja, where historical records were also available (Barbadillo & García-París, 1991; I. Esteban, pers. comm.).

The decline of *A. obstetricans* in the study area seems to be directly related to fish introductions. The lack of evidence of normal reproductive activity in fish-stocked ponds that were formerly occupied by the species, together with the fact that they are locally abundant in fishless streams both suggest a negative interaction between fish and *A. obstetricans*. The absence of larvae of *A. obstetricans* in ponds occupied by fish might be better explained by direct avoidance of reproduction sites with high densities of predatory fish by adult toads than by direct predation of fish on larvae. High densities of larval *A. obstetricans* were observed in some fishless ponds and streams in the study area. Declines of *A. obstetricans* related to other causes (red-leg disease and chytrid fungi) have been reported in other high altitude populations (Márquez *et al.*, 1995; Bosch *et al.*, 2001). In any case, there is no evidence that these pathogens might be involved in the decline of the species at Neila. In-depth analyses regarding other possible factors affecting *A. obstetricans* populations are needed in order to clarify the situation of this species at the area.

In general, introduced fish species (with 25 species reported in Spain: Elvira, 2001) are a problem for native amphibian populations. Introductions were initially carried out by the authorities in the first half of the last century for sport fishing. Although the negative effect of introduced fish has long been identified and stressed (Elvira, 2001), few efforts have been made to control or eradicate any of the species involved. In areas where massive introductions of fish have taken place, amphibian populations are strongly dependent on ephemeral ponds, and these are subject to greater risks of recruitment failure as they are more unpredictable environments than permanent ponds. Moreover, some species that are linked to permanent sites might be rapidly declining due to their inability to respond to new predation pressures, resulting in a pessimistic future. However, adequate management decisions might avert this situation, because it has been shown that amphibian populations affected by fish introductions may recover to pre-stocking levels within a reasonable period of time (>10 years) (Knapp *et al.*, 2001). As these authors conclude, a key point in achieving this recovery is ensuring that measures are put in practice before connectivity to nearby populations falls below a critical threshold, so that recolonization is still possible.

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## REFERENCES

Alford, R. A. & Richards, S. J. (1999). Global amphibian declines: a problem in applied ecology. *Annual Review of Ecology and Systematics* **30**, 133-165.

Aronsson, S. & Stenson, J. A. E. (1995). Newt-fish interactions in a small forest lake. *Amphibia-Reptilia* **16**, 177-184.

Barbadillo, L. J. (1987). *La Guía de Incafo de los anfibios y reptiles de la Península Ibérica, islas Baleares y Canarias*. Incafo, Madrid, Spain.

Barbadillo, L. J. & García-París, M. (1991). Problemas de conservación de los anfibios en España. *Quercus* **62,** 20-25.

Barbadillo, L. J. & Sánchez-Herráiz, M. J. (1998). Reserva de caza de las Sierras de la Demanda y Neila (sector burgalés). In: *Inventario de las áreas importantes para los anfibios y reptiles de España,* 109-114. Santos, X.; Carretero, M. A.; Llorente, G. A. & Montori, A. (Eds.). Ministerio de Medio Ambiente, Madrid, Spain.

Barbadillo, L. J., Lacomba, I., Pérez-Mellado, V., Sancho, V. & López-Jurado, L. F. (1999). *Anfibios y Reptiles de la Península Ibérica, Baleares y Canarias*. Geoplaneta, Barcelona, Spain.

Bosch, J., Martínez-Solano, I. & García-París, M. (2001). Evidence of a chytrid fungus infection involved in the decline of the common midwife toad (*Alytes obstetricans*) in protected areas of central Spain. *Biological Conservation* **97,** 331-337.

Bradford, D. F. (1989). Allotopic distributions of native frogs and introduced fishes in high Sierra Nevada lakes of California: implication of the negative effect of fish introductions. *Copeia* **1989**, 775-778.

Bradford, D. F. (1991). Mass mortality and extinction in a high-elevation population of *Rana muscosa*. *Journal of Herpetology* **25,** 174-177.

Braña, F., Frechilla, L. & Orizaola, G. (1996). Effect of introduced fish on amphibian assemblages in mountain lakes of northern Spain. *Herpetological Journal* **6**, 145-148.

Brönmark, C. & Edenham, P. (1994). Does the presence of fish affect the distribution of tree frogs (*Hyla arborea*)? *Conservation Biology* **8**, 841-845.

Drost, C. A. & Fellers, G. M. (1996). Collapse of a regional frog fauna in the Yosemite area of the California Sierra Nevada, USA. *Conservation Biology* **10**, 414-425.

Elvira, B. (2001). Peces exóticos introducidos en España. In *Atlas y Libro Rojo de los peces continentales de España,* 267-272. Doadrio, I. (Ed.). Dirección General

de Conservación de la Naturaleza – Museo Nacional de Ciencias Naturales, Madrid, Spain.

Fisher, R. N. & Shaffer, H. B. (1996). The decline of amphibians in California's Great Central Valley. *Conservation Biology* **10**, 1387-1397.

Galán, P. (1997). Declive de poblaciones de anfibios en dos embalses de La Coruña (noroeste de España) por introducción de especies exóticas. *Boletín de la Asociación Herpetológica Española* **8**, 38-40.

Gamradt, S. C. & Kats, L. B. (1996). Effect of introduced crayfish and mosquitofish on California newts. *Conservation Biology* **10**, 1155-1162.

Hecnar, S. J. & M'Closkey, R. T. (1997). The effects of predatory fish on amphibian species richness and distribution. *Biological Conservation* **79**, 123-131.

Heyer, W. R., Donnelly, M. A., McDiarmid, R. W., Hayek, L. C. & Foster, M. S. (1994). *Measuring and Monitoring Biological Diversity. Standard Methods for Amphibians*. Smithsonian Institution Press. London, UK.

Kats, L. B., Petranka, J. W. & Sih, A. (1988). Antipredator defenses and the persistence of amphibian larvae with fishes. *Ecology* **69**, 1865-1870.

Kiesecker, J. M., Blaustein, A. R. & Miller, C. L. (2001). Transfer of a pathogen from fish to amphibians. *Conservation Biology* **15**, 1064-1070.

Knapp, R. A., Matthews, K. R. & Sarnelle, O. (2001). Resistance and resilience of alpine lake fauna to fish introductions. *Ecological Monographs* **71**, 401-421.

Lizana, M. & Barbadillo, L. J. (1997). Legislación, protección y estado de conservación de los anfibios y reptiles españoles. In: *Distribución y biogeografía de los anfibios y reptiles en España y Portugal*, 477-516. Pleguezuelos, J. M. (Ed.). Granada: AHE-Universidad de Granada, Spain.

Llorente, G. A. & Arano, B. (1997). *Rana perezi* (Seoane 1895). In *Distribución y Biogeografía de los Anfibios y Reptiles en España y Portugal*, 164-166. Pleguezuelos, J. M. (Ed.). Granada: AHE-Universidad de Granada, Spain.

Manteifel, Y. (1995). Chemically-mediated avoidance of predators by *Rana temporaria* tadpoles. *Journal of Herpetology* **29**, 461-463.

Márquez, R., Olmo, J. L. & Bosch, J. (1995). Recurrent mass mortality of larval midwife toads *Alytes obstetricans* in a lake in the Pyrenean mountains. *Herpetological Journal* **5** 287-289.

Miaud, C. (1995). Oviposition site selection in three species of European newts (Salamandridae) genus *Triturus*. *Amphibia-Reptilia* **16**, 265-272.

Moravec, J. (1989). Observation on fertilization and oviposition in the European tree frog (*Hyla arborea*). *Acta Universitatis Carolinae Biologica* **32**, 515-530.

Petranka, J. W., Kats, L. B. & Sih, A. (1987). Predator-prey interactions among fish and larval amphibians: use of chemical cues to detect predatory fish. *Animal Behaviour* **35**, 420-425.

Woodward, B. D. (1983). Predator-prey interactions and breeding-pond use of temporary-pond species in a desert anuran community. *Ecology* **64**, 1549-1555.

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# BUCCAL SWABS AS A NON-DESTRUCTIVE TISSUE SAMPLING METHOD FOR DNA ANALYSIS IN AMPHIBIANS

NATHALIE PIDANCIER[1], CHRISTIAN MIQUEL[1], CLAUDE MIAUD[2]

[1]*UMR CNRS 5553, Laboratoire de Biologie des Populations d'Altitude, Université Joseph Fourier BP53, 38041 Grenoble, France*

[2]*UMR CNRS 5553, Laboratoire de Biologie des Populations d'Altitude, Université de Savoie, 73 376 Le Bourget du Lac, France*

This study describes a non-destructive DNA sampling method for genetic studies on amphibians using buccal swabs. We assessed the quantity and quality of DNA collected in each species by amplifying a part of the cytochrome *b* gene (381-1060 bp) and microsatellite markers. Buccal swab sampling is a useful alternative method for DNA sampling for both mtDNA and nDNA markers in amphibians. However, only frozen storage allowed microsatellite genotyping. We conclude that this method could greatly increase the accessibility of genetic studies in small vertebrates and could be preferred in the field of conservation genetics.

*Key words*: sampling, mtDNA, nDNA, conservation genetics

## INTRODUCTION

Non-invasive and non-destructive tissue sampling methods for DNA analysis are preferred in the field of conservation genetics. Non-destructive sampling involves the catching of animals to obtain samples (tissue, biopsy) for genetic analysis, whereas with non-invasive methods samples are collected without the need for animal contact (Taberlet *et al.*, 1999). Non-invasive sampling of hair, faeces, feathers or sloughs is currently used for molecular genetic studies of endangered species. This kind of genetic sampling is less stressful for animals than the non-destructive sampling of blood or tissues. Although non-invasive sampling may limit the number of subsequent genetic applications, it is often appropriate for protected, vulnerable or endangered species. Non-destructive methods are currently used for all vertebrates but non-invasive strategies are restricted to mammals (e.g. hair, faeces) and birds (feathers). Sloughs in reptiles and shed skins in amphibians can be found in the field and used as sources of DNA, but because the availability of these tissues is often low, they are of limited value for most studies.

Obtaining DNA from amphibians has until now only been possible with invasive methods, but recently Davis *et al.* (2002) proposed another method to obtain DNA from amphibian skin secretions. Common practices are to take dead or living individuals (eggs, larvae, juveniles or adults) or tissue samples from individuals (e.g. toe-clip, tail-clip, crest-clip). Tissue sampling causes disturbance and stress to animals, and may also affect subsequent survival rates, breeding behaviour, and reproductive success (Clarke, 1972; Golay & Durrer, 1994; Van Gelder & Strijbosch, 1996; Arntzen *et al.*, 1999). When sampling endangered, vulnerable, or declining amphibian species it is preferable to use the least destructive and least invasive method whenever possible. We tested a novel method, isolating DNA from both anuran and urodele species, using tissues obtained non-destructively with a buccal swab. The quantity and quality of the isolated DNA was determined, and how this was affected by storage conditions of the buccal swabs.

*Correspondence*: C. Miaud, UMR CNRS 5553, Laboratoire de Biologie des Populations d'Altitude, Université de Savoie, 73 376 Le Bourget du Lac, France. *E-mail*: claude.miaud@univ-savoie.fr

## MATERIALS AND METHODS

### SAMPLE COLLECTION AND PROCESSING

Buccal cells were taken using swabs with a plastic tip (14.5 cm) and a cotton bud (length 13.5 mm and width 3 mm). These swabs are commercially available in sterile individual package. Buccal samples were taken for three anuran (*Bufo bufo*, *Rana temporaria* and *Rana esculenta* sk.) and three urodele species (*Triturus cristatus*, *Salamandra salamandra* and *Salamandra atra*).

Collecting buccal cells requires catching and handling the animals. Each collector must choose the least stressful catching and handling method according to the species studied. It was relatively easy to open the mouths of the larger species used in this study (i.e. the frogs *R. esculenta*, *R. temporaria*, toad *B. bufo* and salamander *S. salamandra*) by levering open the upper and lower jaw with a rigid sterile plastic tape. In smaller species (*S. atra* and *Triturus cristatus*), we used a smaller tape. A very limited amount of bleeding sometimes occurred during mouth sampling. The method had to be adapted to each species according to its size, mouth anatomy and behaviour.

Three samples were taken from each animal over an eight hour period. The first swab (fresh sample) was immediately used for a DNA genomic extraction. The second swab was stored for nine weeks at room temperature (room temperature sample) and the third swab was stored at –18°C for nine weeks (frozen sample) before DNA extraction. After sampling, the swabs were put in a

TABLE 1. Mitochondrial primers used and microsatellite markers tested. For the cytochrome *b* sequences, length of the amplified fragment is indicated between brackets. [a] personal primer (available on request to the authors), [b] Taberlet *et al.*, 1992, [c] Kocher *et al.*, 1989, [d] Irwin *et al.*, 1991.

| Species | Mitochondrial primers | Microsatellite locus studied |
|---|---|---|
| *Rana temporaria* | CytbF[a] H15573[b] (421 bp) | RtU4 (Berlin *et al.*, 2000) |
| *Rana esculenta* | CytbF[a] H15573[b] (421 bp) | RlCA1 (Garner *et al.*, 2000) |
| *Bufo bufo* | CytbF[a] H15573[b] (393 bp) | Bbuμ1 (Scribner *et al.*, 1994) |
| *Triturus cristatus* | CytbF[a] H15573[b] (381 bp) | Tri96b (Jehle *et al.*, 2001) |
| *Salamandra salamandra* | L14841[c] H15915[d] (1050 bp) | No microsatellite loci available |
| *Salamandra atra* | L14841[c] H15915[d] (1000 bp) | No microsatellite loci available |

1.5 ml sterile Eppendorf tube. For comparison with traditional methods, a toe-clip sample was also taken. All animals were kept in captivity for 15 days before release. The DNA genomic extractions were conducted with a Qiagen Tissue Kit following the manufacturer's conditions. DNA was eluted in a 250 μl volume (TE buffer) for all extractions and stored at –18°C.

### DNA Quantification and PCR based Assays

Total DNA was quantified with the Picogreen® dsDNA quantification kit (Molecular Probes). The DNA quantity can be overestimated due to bacterial growth during storage. To test the quality of the DNA in the samples, mitochondrial and nuclear markers were amplified by PCR. Specific primers were used to reveal the presence and the quality of amphibian DNA. For the mitochondrial marker, a fragment of cytochrome b was amplified for all species. The PCR reaction was performed in a 25 μl tube containing 10mM Tris-HCl pH 8.3, 50 mM KCl, 2 mM MgCl2, 0.5 μM of each primer, 0.1 mM of each dNTP, Bovine Serum Albumin (5 μg), AmpliTaq Gold DNA polymerase (0.5 U) and 6-20 ng DNA. The primers used are presented in Table 1. Forty-five cycles were performed with 10 min at 95°C followed by 30s at 95°C, 30s at 45°C (except for Salamandra sp. 60s at 50°C), 40s at 72°C (except for Salamandra sp. 60s) and a 5 min final extension at 72°C. The PCR products were purified with a Qiaquick purification kit (Qiagen) and were double strand sequenced with a Big Dye terminator sequencing Kit (Perkin Elmer).

Microsatellite markers were amplified only for *Bufo bufo*, *R. temporaria*, *R. esculenta* and *T. cristatus* because microsatellite markers are currently still unavailable for the *Salamandra* genus. The microsatellite loci studied are presented in Table 1. The PCR reaction components were the same conditions as for sequencing (see above), and forward primers were labelled with fluorochrome. Forty cycles of amplification were performed and PCR products were run on 6%

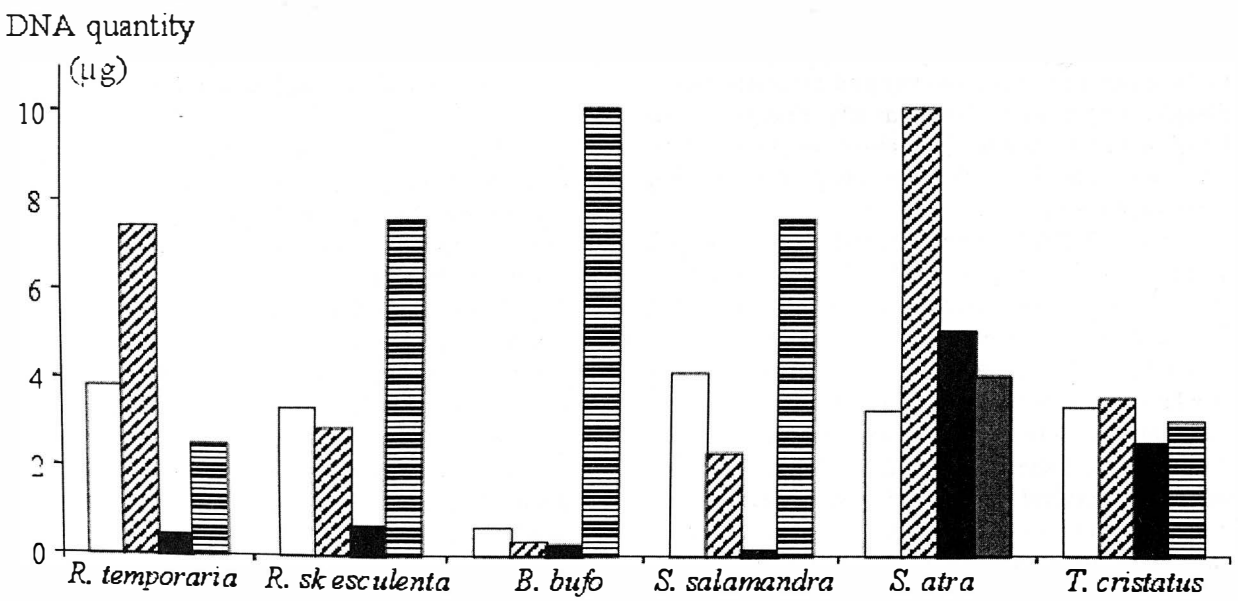


FIG. 1. Total yield of DNA (μg) for buccal swab sampling and toe-clip sampling for six amphibian species (*R. temporaria, R. esculenta* sk., *B. bufo, S. salamandra, S. atra* and *T. cristatus)*. Open bars, fresh samples; oblique shading, frozen samples; filled bars, room temperature; horizontal bars, toe-clips. Fresh samples were immediately extracted after collection. Frozen samples and room temperature samples were stored for 9 weeks at –18°C and at room temperature, respectively, before DNA extraction.

TABLE 2. Results of sequencing and microsatellite genotyping with buccal swabs and toe-clip samples. A successful sequencing or genotyping is indicated by "yes" and failure by "no".

| Species | Cytochrome *b* sequencing | | | | Microsatellite genotyping | | | |
|---|---|---|---|---|---|---|---|---|
| | Buccal swabs | | | Toe-clip | Buccal swabs | | | Toe-clip |
| | Fresh | Room temperature | Frozen | | Fresh | Room temperature | Frozen | |
| *Rana temporaria* | yes | yes | yes | yes | yes | no | yes | yes |
| *Rana esculenta* | yes | no | yes | yes | yes | no | yes | yes |
| *Bufo bufo* | yes | no | yes | yes | yes | no | yes | yes |
| *Triturus cristatus* | yes | yes | yes | yes | yes | no | yes | yes |
| *Salamandra salamandra* | yes | no | yes | yes | - | - | - | - |
| *Salamandra atra* | yes | yes | yes | yes | - | - | - | - |

polyacrylamide gels on an automated ABI 377 DNA sequencer. The allele size was determined with the Genotyper software version 2 (Perkin Elmer).

## RESULTS AND DISCUSSION

As we estimated DNA concentrations using only one individual per species, the values are only indicative. The total DNA yields from the fresh and frozen samples were similar in *R. esculenta*, *S. salamandra*, *B. bufo* and *T. cristatus*, or higher from the frozen samples in *R. temporaria* and *S. atra* (Fig. 1). The total DNA yields from the fresh and frozen samples, were higher than those from the room temperature samples, in four of the six species. Nucleic acid degradation was observed with agarose gel images at room temperature, but was very limited at –18°C. In *Salamandra atra* a higher DNA yield was obtained at room temperature than in the other storage conditions. Bacterial growth and DNA contamination may explain this higher value. Moreover, because amphibians have nucleated red blood cells, the limited bleeding which occurred during the mouth sampling of some individuals could account for the variation in DNA quantity observed among the species.

We successfully amplified parts of the cytochrome *b* gene from fresh and frozen buccal swabs and from the toe-clip samples in all six species. PCR products for the room temperature samples were only obtained from *R. temporaria*, *T. cristatus,* and *S. atra* but not from *R. esculenta, B. bufo,* and *S. salamandra* (Table 2). For each species, the cytochrome *b* sequences obtained were identical across storage conditions and matched with the correct taxon. For microsatellite markers, a correct fluorescent profile was obtained for the four tested species for all samples except for the buccal samples stored at room temperature (Table 2). For each individual, allele sizes were identical with DNA extracted from the fresh and frozen buccal samples and from the toe-clip sample.

As DNA quantity varies between amphibian species, we recommend initiating a pilot study to test the reliability of the buccal swab sampling DNA method before using this method on different species. Moreover, with very low DNA quantity, increasing genotyping errors with microsatellite markers can affect the reliability of genetic analysis (Taberlet *et al.*, 1999), and, a multiple-tube approach (i. e. repeating treatments several times for each locus and each extract) might be recommended to avoid such problems (Goossens *et al.*, 1998).

We did not test the effect of storage buffer on the preservation of DNA. It is sure that a storage buffer could improve DNA preservation by limiting nucleic acid degradation and bacterial growth.

This method for collecting tissue for subsequent amphibian genetic molecular studies presents several positive features compared to sampling methods such as toe-clipping. Buccal swab sampling is easy to perform, few materials are needed, there are no liquids to handle (storage buffer or disinfectant) in the field and it is as cheap as other sampling procedures. Moreover it may be easier to obtain sampling permits, especially for endangered species.

Here, we have shown that it is possible to collect DNA with buccal swabs and to perform subsequent genetic analysis, but there is not sufficient evidence to imply a general applicability of buccal swabs as a DNA source in large scale projects.

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## REFERENCES

Arntzen, J. W., Smithson, A. & Oldham R. S. (1999). Marking and tissue sampling effects on body condition and survival in the newt (*Triturus cristatus*). *Journal of Herpetology* **33**, 567-576.

Berlin, S., Merila, J. & Hellegren H. (2000). Isolation and characterization of polymorphic microsatellite loci in the common frog, *Rana temporaria. Molecular Ecology* **9**, 1938-1939.

Clarke, R. D. (1972). The effect of toe clipping on survival in Fowler's toad (*Bufo woodhousei fowleri*). *Copeia* **1972**, 182-185.

Davis, A. Sluscher, L. B. & Beneski J. T. (2002). A novel method for the extraction and amplification of DNA from amphibians. *Herpetological Review* **33**, 104-105.

Garner, T. W., Gautschi, J. B., Röthlisberger, S. & Reyer H.-U. (2000). A set of CA repeat microsatellite markers derived from the pool frog, *Rana lessonae*. *Molecular Ecology* **9**, 2155-2234.

Golay, N. & Durrer, H. (1994). Inflammation due to toe-clipping in natterjacks toads (*Bufo calamita*). *Amphibia-Reptilia* **15**, 81-83.

Goossens, B., Waits, L. P. & Taberlet P. (1998). Plucked hair samples as a source of DNA: reliability of dinucleotide microsatellite genotyping. *Molecular Ecology* **7**, 1237-1241.

Irwin, D., Kocher, T. & Wilson A. (1991). Evolution of the cytochrome *b* gene of Mammals. *Journal of Molecular Evolution* **32**, 128-144.

Jehle, R., Arntzen, J. W., Burke, T., Krupa, A. P. & Hödl W. (2001). The annual number of breeding adults and the effective population size of syntopic newts (*Triturus cristatus, T. marmoratus*). *Molecular Ecology* **10**, 839-850.

Kocher, T. D., Thomas, W. K., Meyer, A. Pääbo, S., Villablanca, F. X. & Wilson A. C. (1989). Dynamics of mitochondrial evolution in animals: amplification and sequencing with conserved primers. *Proceedings of the National Academy of Sciences of the United States of America* **86**, 6196-6200.

Scribner, K. T., Arntzen, J. W. & Burke T. (1994). Comparative analysis of intra- and interpopulation genetic diversity in *Bufo bufo*, using allozyme, single-locus microsatellite, minisatellite, and multilocus minisatellite data. *Molecular Biology Evolution* **11**, 737-748.

Taberlet, P., Meyer, A. & Bouvet J. (1992). Unusual mitochondrial DNA polymorphism in two local populations of blue tit *Parus caeruleus*. *Molecular Ecology* **1**, 27-36.

Taberlet, P., Waits, L. P. & Luikart, G. (1999). Noninvasive genetic sampling: look before you leap. *Trends in Ecology and Evolution* **14**, 323-327.

Van Gelder, J. J. & Strijbosch H. (1996). Marking amphibians: effects of toe-clipping on *Bufo bufo* (Anura: Bufonidae). *Amphibia-Reptilia* **17**, 169-174.

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# DIFFERENCES IN SIZE AT BIRTH AND BROOD SIZE AMONG PORTUGUESE POPULATIONS OF THE FIRE SALAMANDER, *SALAMANDRA SALAMANDRA*

RUI REBELO AND MARIA HELENA LECLAIR

*Centro de Biologia Ambiental, Departamento de Zoologia e Antropologia, Faculdade de Ciências de Lisboa, Bloco C2, Piso 3, Campo Grande, 1749-016 Lisboa, Portugal*

Size at birth and brood size were studied in ovoviviparous *Salamandra salamandra gallaica* and *S. s. crespoi* from areas differing in hydrological regime. Gravid females were maintained in open-air terraria until parturition was completed. Sizes of offspring at birth tended to be less variable in populations from mesic areas, and brood sizes (numbers of offspring) were larger in a population from a xeric site. Large sizes at birth, close to those observed in viviparous *S. s. bernardezi*, could be attributed not to cold climate, the risk of larval drift or short pond duration, but perhaps to competition or predation by conspecific larvae. Large and small larvae differed in time taken to reach metamorphosis, but not in size at metamorphosis. Females from the xeric site gave birth to large numbers of small larvae, mainly in small groups and on separate occasions. In the wild, this probably results in the dispersal of a female's offspring among several ponds.

*Key words:* fecundity, larval size, Mediterranean climate, metamorphosis, Urodela

## INTRODUCTION

Size at birth is an important component of an individual's life history, as small initial differences in size can become large differences in several life history traits later in life (Roff, 1992; Stearns, 1992). As resources available for reproduction are limited, the investment in large offspring should be counterbalanced by selection favouring the production of the greatest number of progeny. This compromise between quality and quantity of offspring is still one of the central ideas in theories concerning the evolution of size at birth (Smith & Fretwell, 1974; Steams, 1992).

Among other factors, climatic conditions and food availability may select for increased variability in size at birth. However, if the function relating offspring size to survival probability is strong and highly repeatable from year to year, a population-specific optimal size at birth (OSB) may be selected (McGinley, 1989; Shine, 1989; Steams, 1992). If an OSB does exist, most variation in reproductive output due to environmental conditions will be in clutch size rather than offspring size. Thus, some models predict that clutch mass will be related to clutch size, but not to offspring size (Roff, 1992; Roosenburg & Dunham, 1997).

Reproductive characteristics such as egg size, size at birth and clutch size are traditionally seen as highly variable within – as well as between – populations of amphibians (Kaplan & Cooper, 1984). Much of that variability is regarded as an adaptation to unpredictable environments, such as vernal ponds and streams – the major larval habitats for temperate-zone amphibians. Such factors as within-clutch variation in offspring size, platykurtic distributions of egg sizes, or the absence of an OSB, have been seen as an adaptation to unpredictability (Crump, 1981; Kaplan & Cooper, 1984).

Some factors linking urodele larval size to survival have already been identified: (1) Larval drift – as small larvae are more prone to drift (Bruce, 1985), larger sizes at birth should be favoured in running waters (and smaller sizes in still waters). For example, stream-breeding females of *Ambystoma texanum* tend to lay larger eggs than pond-breeding females (Petranka, 1984); in northern Spain, larvae of stream-breeding *Salamandra salamandra bernardezi* are considerably larger than those of pond-breeding *Salamandra s. almanzoris* (Thiesmeier, 1994). (2) Pond or stream duration – ultimately, this determines the amount of time available to grow and metamorphose. Given that age at metamorphosis is usually inversely related to size at birth (Semlitsch, 1987; Rowe & Ludwig, 1991), the shorter the pond duration, the larger the neonates should be. For example, *Ambystoma maculatum* populations that lay in temporary ponds produce larger eggs than those that lay in permanent ponds (Woodward, 1982).

The fire salamander, *Salamandra salamandra* (L.), is a long-lived, iteroparous urodele, widespread in western and southern Europe (Thorn, 1968). Some subspecies or populations, usually from mountain habitats, reproduce biennially and give birth to fully metamorphosed juveniles, while lowland subspecies are usually ovoviviparous and reproduce annually, giving birth to aquatic larvae at different stages of development (Joly *et al.*, 1994). In the Iberian Peninsula, fire salamanders occur from sea level to altitudes of more than 2000 m and from wet temperate forests to arid scrubland and steppe (Pleguezuelos, 1997). South of the river Tejo, the species withstands typical Mediterranean conditions at one of the dry limits of its distribution. The subspecies occurring in Portugal, *Salamandra salamandra gallaica* and *S. s. crespoi* (Fig. 1), are ovoviviparous, giving

*Correspondence:* R. Rebelo, Departamento de Zoologia e Antropologia, Faculdade de Ciências de Lisboa, Bloco C2, Piso 3, Campo Grande, 1749-016 Lisboa, Portugal. *E-mail:* rui.rebelo@fc.ul.pt

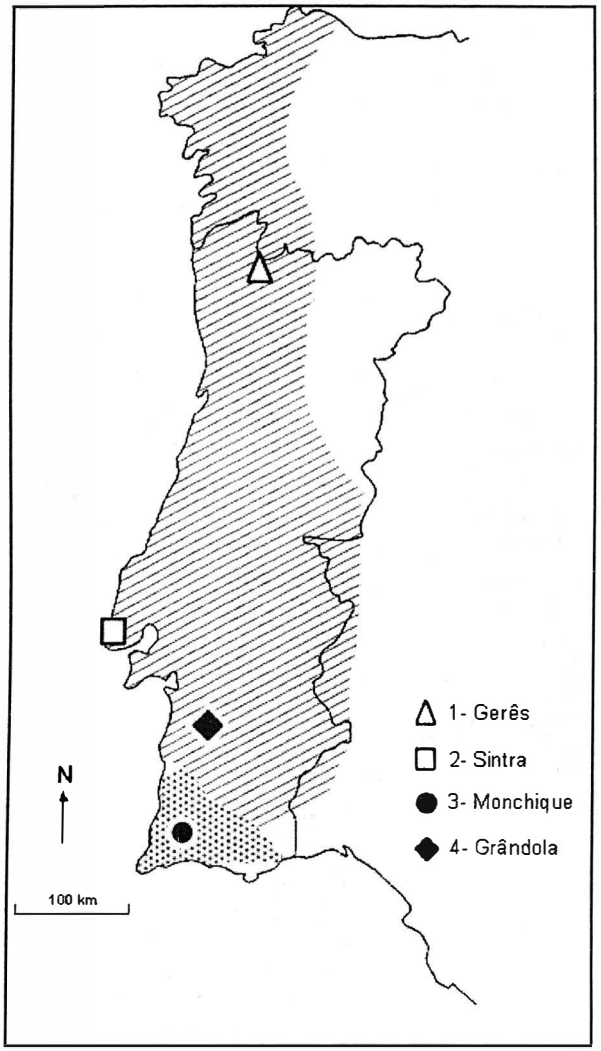


FIG. 1. Study areas: Gerês (1), Sintra (2), Monchique (3), and Grândola (4). Shaded area, *Salamandra salamandra gallaica* (adapted from Thorn, 1968); stippled area, *S. salamandra crespoi* (adapted from Malkmus, 1983).

birth to well-developed larvae in temporary ponds or streams between November and April. Reproduction is probably annual, as in southern France (Joly *et al.*, 1994). Larvae are born inside the transparent egg capsule and hatch immediately after contact with water (R. Rebelo, *pers. obs.*). The larval period may vary from one to six months (Barbadillo, 1987). Studies addressing the reproductive traits of *S. salamandra* in Iberia have focused on the viviparous subspecies, *S. s. bernardezi* and *S. s. fastuosa* (Dopazo & Alberch, 1994; Thiesmeier *et al.*, 1994).

We studied four populations of the fire salamander living in regions with marked differences in hydrological regimes. Our aim was to examine the habitat influences on size at birth. We investigated the following: (1) the influence of maternal body size, season and year on size at birth; (2) the trade-off, if any, between brood size and neonate size; (3) the existence of an optimal size at birth; (4) the relationship between size at birth and both size at metamorphosis and length of larval period.

## STUDY AREAS AND METHODS

We sampled four areas arranged along a north-south axis (Fig. 1), which encompass much of the environmental variability withstood by the subspecies *S. s. gallaica* and *S. s. crespoi*. The extent and altitude of the mountain ranges on which the study areas are located decrease from area 1 to area 4: area 1 is subjected to more mesic conditions, while xeric conditions become dominant in area 4 (Table 1). Area 1 (Gerês) is a climax deciduous oak forest (mainly *Quercus robur* and *Q. pyrenaica*) on Gerês Mountain. Area 2 (Sintra) is on a plateau covered with introduced *Acacia* sp. Area 3, Monchique, is the highest mountain in southern Portugal, constituting an ecological island of mesic, wet climate in an otherwise Mediterranean, flat and dry region, as typified by area 4. In this area, salamanders were caught in valleys covered with *Q. canariensis*, *Q. suber* and introduced *Acacia* sp. Area 4 (Grândola), is a flat area located at the base of

TABLE 1. Physical and climatic parameters of the study areas. Data were obtained from the "Atlas do Ambiente" (C. N. A., 1983): Pond/stream duration from 1992/93 to 1997/98 as follows: pond filling - pond drying month (no. months with water). No visits were made during 1996/97.

| | Gerês (1) | Sintra (2) | Monchique (3) | Grândola (4) |
|---|---|---|---|---|
| Altitude (m) | 550 | 450 | 500 | 100 |
| Maximum altitude (m) | 1508 | 490 | 902 | 326 |
| Slope (%) | >35 | 8-15 | 15-25 | 4-8 |
| Mean annual temperature (°C) | 7.5/10 | 12.5/15 | 15 | 17 |
| Mean annual precipitation (mm) | >2800 | 1000/1200 | 1000/1200 | 600/800 |
| Pond/stream duration 1992/93 | Oct-May (8) | Oct-Mar (6) | Oct-Mar (6) | – |
| Pond/stream duration 1993/94 | Oct-Apr (7) | Nov-Mar (5) | Nov-Feb (4) | Nov-Jan (3) |
| Pond/stream duration 1994/95 | Oct-Apr (7) | Nov-Mar (5) | Nov-Mar (5) | Nov-Mar (5) |
| Pond/stream duration 1995/96 | Oct-May (8) | Nov-Mar (5) | Nov-Mar (5) | Nov-Feb (4) |
| Pond/stream duration 1997/98 | no data | Nov-Mar (5) | no data | Nov-Jan (3) |

the Grândola Hills, covered with an open forest of cork oak (*Q. suber*).

As in other Mediterranean regions, the climate in Portugal is characterized by long, hot summers, during which almost all ponds and streams dry up. The first rains, usually in October, mark the beginning of the activity period for the fire salamander, which extends until April/May. From 1992 to 1996, the study areas were visited at least once a month during the salamander activity period. As part of another study, Sintra and Grândola were also visited until 1998. Ponds and streams were checked first for the presence of water and then for larvae or pre-metamorphs (characterized by the typical adult yellow-and-black pattern, together with reducted gills).

Gravid females were found crossing unpaved forest roads. Captures were made in early autumn (October), before the filling of ponds or streams. Gerês (1) was sampled in 1994/95, Sintra (2) in 1994/95 and 1995/96, and Monchique (3) and Grândola (4) in 1995/96. Altogether, 64 females were caught at the beginning of the rainy season. During 1995/96, 18 additional females from Sintra and Grândola were caught during the monthly visits, well after the beginning of the rains. Females were measured (snout-vent length – SVL), weighed and photographed dorsally in order to allow subsequent individual recognition. We also collected females that were found dead during the autumnal visits – mostly animals that had been run over by vehicles on paved roads. In the dead animals, we counted the number of ovarian follicles larger than 2 mm diameter (Greven & Guex, 1994)-roughly corresponding to stages V and VI of oocyte maturation, already described for another fire salamander subspecies, *S. s. infraimmaculata* (Sharon, *et al.*, 2000).

The live females were kept at the Faculty of Sciences, Lisbon, in individual, open-air terraria (50 × 30 × 30cm) provided with small pools. Food (*Tenebrio molitor* larvae and earthworms) was provided *ad libitum*, as the parturition period can last up to one month. Terraria were checked every morning for larvae, which were measured to the nearest mm (total length and SVL), weighed to the nearest mg ,and moved to a different container (one container per brood). Most of the data concern the larvae produced by the 64 females maintained from the beginning of the rainy season. For these animals it was possible to calculate relative brood mass (RBM), i.e. brood mass divided by female initial mass. Late-season females from Sintra and Grândola also gave birth to some larvae, which were considered as partial broods and used for some analyses if at least two larvae were obtained (this is the reason for some differences in sample sizes). Nine females were killed after their residence in the terraria, and their uterine contents were examined. Larvae and females were released at the place of capture at the end of the study.

In order to assess the effect of size at birth on length of the larval period and size at metamorphosis, seven similar-sized larvae from each of five broods from Sintra and four broods from Grândola were raised at the bioterium of the Faculty of Sciences. Larvae were kept under natural photoperiod in individual, 250 ml containers filled with pond water. Water temperatures fluctuated daily at 20±2 °C. Every other day, water was changed and larvae were fed *ad libitum* with minced beef. At the completion of metamorphosis, individuals were measured and released. Due to the long larval periods, the experiment was concluded for each brood when four of the initial seven larvae completed metamorphosis. Larval period was recorded from the date of birth to that of metamorphosis. Mean growth rate during the larval period was calculated as [(size at metamorphosis – size at birth)/ larval period].

### Statistical Analyses

To avoid statistical non-independence of the data from siblings, we considered each brood as the sampling unit and used mean values for each trait within each brood.

The Chi-square test was used to compare absolute frequencies. After checking for normality and homogeneity of variances (Levene's test), a *t*-test or one-way ANOVA was used for the majority of comparisons; the Tukey HSD for unequal *N* was the *a posteriori* test used. Whenever ANOVA assumptions were not met, a non-parametric test was used (Kruskal-Wallis ANOVA). Correlations among variables were determined with Pearson's product-moment correlation coefficient. For correlations involving temporal parameters, the first of November was chosen as day 1, as no larvae were born before that day. Analyses were performed with STATISTICA for Windows, Release 5.0 (StatSoft, Inc. 1995).

## RESULTS

### Study Areas

Gerês is crossed by many rapid streams, owing to the steep slopes and abundant rainfall. Larvae were found mainly in the quieter sections of the streams, but also in some temporary ponds. This was the only area that maintained free water in small streams during all the year. In Sintra and Monchique, the few streams dried quickly and larvae were mostly found in springs and wells, and in the water of underground mines. Ponds and slow-moving streams were abundant during the rainy season in Grândola, but dried quickly during the spring (with considerable annual variation). In two of the study years (1993/94 and 1997/98), the lack of January rains led to the early drying of all the water bodies and very few larvae were observed after the spring rains in March. Considering all the study years, and as would be expected, pond duration decreased from area 1 to area 4, while between-year variation increased (Table 1). Except for two single cases in Gerês, no larvae were found after May.

TABLE 2. Reproductive characteristics of female salamanders. Data in each case are: top row, mean ± standard deviation, except for the number of parturition episodes and parturition period, where the median is presented; middle row, range; bottom row, sample size. * $P<0.05$; ** $P<0.01$; *** $P<0.001$. Values in the same row with different superscripts are significantly different.

| | Gerês (1) | Sintra (2) | Monchique (3) | Grândola (4) |
|---|---|---|---|---|
| Female SVL (mm) | 96.16±6.27 $^{a***}$<br>(86.5-107.5)<br>$n$=24 | 101.91±7.73 $^{ac**}$<br>(91.1-112.75)<br>$n$=24 | 107.11±7.54 $^{bc}$<br>(92.4-116)<br>$n$=17 | 111.97±10.31 $^{b}$<br>(93.5-128.4)<br>$n$=11 |
| Relative brood mass (%) | 0.168±0.064 $^{a*}$<br>(0.078-0.318)<br>$n$=13 | 0.159±0.049 $^{a*}$<br>(0.086-0.247)<br>$n$=15 | 0.195±0.058<br>(0.114-0.282)<br>$n$=10 | 0.247±0.088 $^{b}$<br>(0.170-0.416)<br>$n$=6 |
| No. of parturition nights | 3<br>(1-7)<br>$n$=11 | 2<br>(1-10)<br>$n$=14 | 3<br>(2-7)<br>$n$=9 | 8<br>(2-12)<br>$n$=5 |
| Parturition period (days) | 5<br>(1-24)<br>$n$=11 | 8<br>(1-28)<br>$n$=14 | 7<br>(5-18)<br>$n$=9 | 19<br>(2-43)<br>$n$=5 |

### FEMALE CHARACTERISTICS

Female body size differed among the populations ($F_{3,72}$=12.75, $P<0.001$), increasing from area 1 to area 4 (Table 2). Some females did not give birth to any larvae (1, 0, 3 and 2 females from areas 1 to 4, respectively), while others (4, 2, 6 and 2 females, respectively) produced only 1 to 5 larvae. The proportion of such females did not differ among populations ($\chi^2$=5.76, df=3, $P>0.05$) and they were excluded from some analyses.

Two out of the nine females that were killed at the end of the study had not given birth to any larvae, and their uteri were empty. Of the remaining seven females, five had empty uteri and the remaining two still had some larvae (1 and 4, respectively) in the uteri; the unborn larvae were smaller than the larvae that had been born previously .

There were significant differences in relative brood mass (RBM) among the populations ($F_{3,40}$=3.10, $P<0.05$), salamanders at Gerês and Sintra had smaller RBMs than those at Grândola (Table 2). There was no statistically significant correlation between RBM and female SVL at any site.

When giving birth, different strategies were employed by different females, even within each population: e.g. releasing the entire brood in 2-3 nights within one week and releasing just a few larvae at a time, over more than one month. Overall, both number of parturition episodes and parturition period were highest among salamanders from Grândola (Table 2); however, differences among populations were not statistically significant (Kruskal-Wallis tests: $H_{3,39}$=5.56, $P$=0.13 for number of parturition nights; $H_{3,39}$=5.43, $P$=0.14 for parturition period). Neither of these traits was correlated with either brood mass or brood size in any of the populations.

### BROODS AND LARVAE

Log-transformed brood size and ovarian follicle counts differed among populations ($F_{3,40}$=22.32, $P<0.001$; $F_{3,28}$=8.76, $P<0.01$ respectively), and both were significantly larger in Grândola salamanders (Table 3). The smallest follicle counts and the smallest brood sizes were found at Sintra. In no case was log-transformed brood size correlated significantly with female SVL.

In total, 2205 larvae were born in captivity. Larval length and mass at birth were strongly correlated ($r$=0.907, $P<0.0001$). Within-brood larval size variation was small, regardless of the population (Table 3). In the majority of broods, whatever the population, larval body mass was normally distributed. In a few broods the distribution was leptokurtic (Table 3); the number of such broods did not differ among sites ($\chi^2$=5.38, df=7, $P<0.61$).

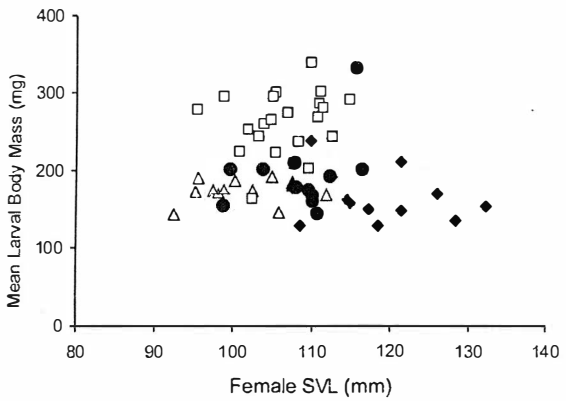


FIG. 2. Mean larval body mass at birth as a function of female body size in *Salamandra salamandra gallaica* and *S. crespoi* at four sites in Portugal: Gerês (1), Sintra (2), Monchique (3) and Grândola (4). Data concern all broods (complete and partial). Symbols as in Fig. 1.

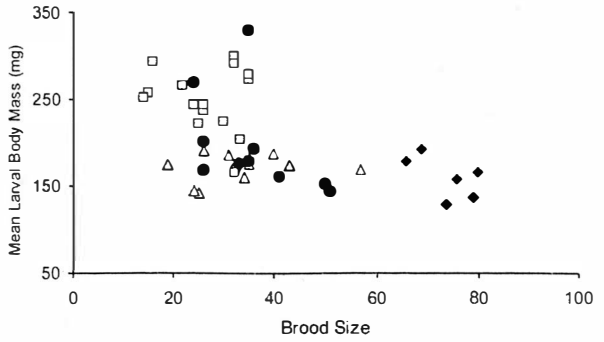


FIG. 3. The relationship between mean larval body mass at birth and brood size in *Salamandra salamandra gallaica* and *S. salamandra crespoi* at four sites in Portugal: Gerês (1), Sintra (2), Monchique (3), Grândola (4).Symbols as in Fig. 1.

There were significant between-population differences in larval body mass ($F_{3,63}$=8.76, $P$<0.001) and larval length ($F_{3,63}$=18.32, $P$<0.001). Salamanders from Sintra had the largest larvae, while very small larvae were mainly found in salamanders from Gerês and Grândola (Table 3). None of the within-population correlations between mean larval mass and female SVL was significant (Fig. 2). A negative correlation between brood size and mean larval mass was found only at Monchique ($r$=-0.620, $n$=10, $P$<0.05; Fig. 3).

To search for evidence of population-specific optimal size at birth, we compared the coefficients of variation for both brood size and larval size at birth, and looked for positive correlations between brood mass and brood size. For the northern populations (1 and 2), brood size varied almost twice as much as larval size at

TABLE 3. Brood and larval characteristics: top row, mean ± standard deviation; middle row, range; bottom row, sample size. * $P$<0.05; ** $P$<0.01; *** $P$<0.001; NS, not significant. Sample sizes differ whenever data from partial broods were included and for wild SVL at metamorphosis. Values in the same row with different superscripts are significantly different.

| | Gerês (1) | Sintra (2) | Monchique (3) | Grândola (4) |
|---|---|---|---|---|
| Brood size | 33.5±9.7$^{a}$<br>(19-57)<br>$n$=13 | 26.5±7.2$^{a}$<br>(14-35)<br>$n$=15 | 35.7±9.4$^{a}$<br>(24-51)<br>$n$=10 | 74.0±5.5$^{b***}$<br>(66-80)<br>$n$=6 |
| No. of ovarian follicles | 52.1±11.5$^{a}$<br>(37-75)<br>$n$=11 | 33.5±12.1$^{a}$<br>(18-53)<br>$n$=9 | 50.0±11.6$^{a}$<br>(34-62)<br>$n$=7 | 89.6±43.3$^{b***}$<br>(43-143)<br>$n$=5 |
| Larval total length at birth (mm) | 30.3±2.1$^{a}$<br>(25-31.96)<br>$n$=15 | 34.2±1.6$^{b***}$<br>(31-35.5)<br>$n$=23 | 31.3±2.7$^{a}$<br>(27-38)<br>$n$=13 | 29.3±2.6$^{a}$<br>(23-32.4)<br>$n$=16 |
| Larval mass at birth (mg) | 167±22$^{a}$<br>(120-190)<br>$n$=15 | 263±41$^{b***}$<br>(164-337)<br>$n$=23 | 198±51$^{a}$<br>(143-331)<br>$n$=13 | 164± 34$^{a}$<br>(105-238)<br>$n$=16 |
| Within-brood coefficient of variation of larval mass at birth | 0.11±0.02<br>(0.06-0.15)<br>$n$=13 | 0.12±0.05<br>(0.05-0.17)<br>$n$=15 | 0.09±0.03<br>(0.06-0.12)<br>$n$=10 | 0.10±0.03<br>(0.08-0.16)<br>$n$=6 |
| Among-broods coefficient of variation of larval mass | 13.17<br>$n$=15 | 15.59<br>$n$=23 | 25.76<br>$n$=13 | 20.73<br>$n$=16 |
| Coefficient of variation of brood size | 28.92<br>$n$=13 | 27.05<br>$n$=15 | 26.41<br>$n$=10 | 7.5<br>$n$=6 |
| Correlation ($r$) between brood size and brood mass | 0.85*** | 0.84*** | 0.37 NS | 0.06 NS |
| No. of leptokurtic broods | 1<br>$n$=13 | 3<br>$n$=15 | 3<br>$n$=10 | 4<br>$n$=6 |
| SVL at metamorphosis in the wild (mm) | 25.2±6.5<br>(15-37)<br>$n$=21 | 25.7±2.0<br>(22-28)<br>$n$=23 | 27.2±2.9<br>(23-32)<br>$n$=13 | 26.5±1.8<br>(24-30)<br>$n$=22 |

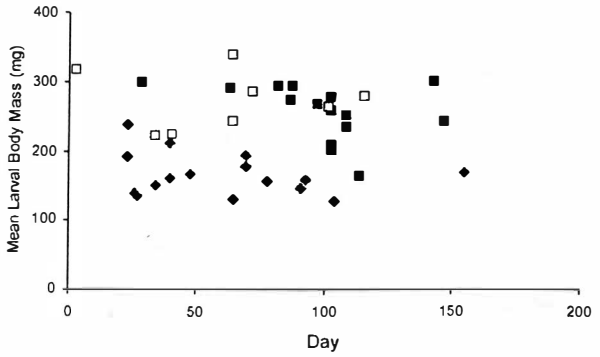


FIG. 4. Mean larval mass at birth as a function of the parturition date of the first larva of each brood (day 1 = 1 November). Data were collected at Sintra (2) in 1994/95 (filled squares); and in 1995/96 (open squares), and at Grândola (4) in 1995-96 (lozenges).

birth and brood mass was correlated with brood size; for the southern populations (3 and 4), larval size at birth varied as much as, or even more than, brood size and there was no correlation between brood mass and brood size (Table 3).

### SEASONAL AND ANNUAL VARIATION

Female SVL was not correlated with date of parturition in any area. Also, there was no significant correlation between mean larval mass and date of parturition, or between the coefficient of variation of larval mass and date of parturition, for any population (Fig. 4). Mean larval mass at Sintra did not differ between 1994/95 and 1995/96 (258±40 mg and 272±42 mg, respectively; $t_{21}$=-0.763, $P$=0.45; Fig. 4).

### METAMORPHOSIS

The largest and smallest sizes at metamorphosis in the wild were observed at Gerês (Table 3). However, the metamorphic size range was generally narrow, and overall no significant difference was found among the populations (Kruskal-Wallis test, $H_{3,85}$=2.35, $P$=0.50).

Larvae from Sintra and Grândola were reared in the laboratory and the results of the experiment are depicted in Table 4. Despite the significant size differences at the beginning of the experiment ($t_7$=7.13, $P$<0.001), SVL at metamorphosis was not different between the two populations ($t_7$ = 0.826, $P$>0.05). However, there was a very significant difference in length of larval period for the first larva to metamorphose ($t_7$=-6.61, $P$<0.001), as well as for the fourth ($t_7$=-8.4, $P$<0.001). Overall, there were no population differences in the mean daily growth rates ($t_7$=0.916, $P$=0.11). Wild and laboratory-reared metamorphs from Sintra were similar in size ($t_{32}$=0.465, $P$=0.65), but laboratory-reared animals from Grândola were somewhat smaller than wild metamorphs (24.5±1.17 versus 26.5±1.77, respectively; $t_{24}$=2.151, $P$=0.046).

## DISCUSSION

Maintaining animals in captivity until the completion of reproduction is time- and space-consuming, but is a non-invasive method for determining clutch size. This method has already been used in some urodeles (e.g. Baker, 1992), including other subspecies of the fire salamander (Dopazo & Alberch, 1994; Degani & Warburg, 1995). However, one must be sure that animals were caught at the beginning of the reproductive period. In fact, the small broods found for some animals may indicate that they had already begun giving birth when

TABLE 4. Results of the larval rearing experiment. Seven larvae from each of five broods from Sintra (2) and four broods from Grândola (4) were reared. The experiment was concluded with the metamorphosis of the fourth larva from each brood. Length of the larval period is provided for the first and fourth l. *** $P$<0.001.

| Broods | Mean SVL at birth (mm) ($n$=7) | Mean SVL at metamorphosis (mm) ($n$=4) | Larval period –1st larva (days) | Larval period – 4th larva (days) | Mean growth rate (µm/day) ($n$=4) |
|---|---|---|---|---|---|
| Sintra (2) | | | | | |
| 1 | 21.71 | 26 | 50 | 71 | 65.5 |
| 2 | 20.91 | 23.75 | 35 | 69 | 53.4 |
| 3 | 20.37 | 23.75 | 49 | 61 | 60.5 |
| 4 | 19.85 | 26.5 | 51 | 89 | 104.8 |
| 5 | 19.38 | 26.25 | 67 | 93 | 81.2 |
| Mean ± SD | 20.4±0.91*** | 25.3±1.38 | 50.4±11.35*** | 76.6±13.74*** | 73.1±20.46 |
| Grândola (4) | | | | | |
| 1 | 17.48 | 26 | 121 | 140 | 61.4 |
| 2 | 15.74 | 24.75 | 92 | 157 | 73.6 |
| 3 | 15.6 | 23.25 | 136 | 149 | 53.4 |
| 4 | 15.81 | 24 | 114 | 135 | 63.6 |
| Mean ± SD | 16.2±0.89*** | 24.5±1.17 | 115.7±18.3*** | 142.3±9.74*** | 63.0±8.32 |

captured (and this is more probable for Gerês, as it is the only area that maintains some free water throughout the year). It is also possible that not all females reproduce every year, as about 10% of the whole sample did not bear any larvae.

Reproduction in the fire salamander is notoriously variable. This may be explained by subspecific differences, and also by inter-population variations within subspecies (Dopazo & Alberch, 1994; Veith, 1994). The number and distribution of fire salamander subspecies in the Iberian Peninsula is still an unresolved question. Recent studies agree on some degree of genetic distinctiveness for south-western populations, including *S. s. crespoi*, but not for the population from Grândola (García-Paris *et al.*, 1998; Steinfarz *et al*, 2000). However, reproductive traits from the Monchique population were not particularly different from those of *S. s. gallaica*. Overall, values of RBM, brood size, size at birth, number of parturition episodes and parturition period of the studied populations were not dissimilar to those of other ovoviviparous populations of the fire salamander from central Europe (Thiesmeier, 1992; Thiesmeier, 1994), the Iberian Peninsula (Barbadillo, 1987; Lizana *et al.*, 1989) and Israel (Warburg, 1992; Degani & Warburg, 1995). There were some exceptions, particularly at Grândola, where brood sizes, RBM, parturition period and number of parturition episodes were relatively large. Larger brood sizes (up to 192) have been reported only in *S. s. infraimmaculata* in Israel ( Degani & Warburg, 1995). Large sizes at birth at Sintra were also an exception, slightly overlapping those of the viviparous *S. s. bernardezi* (Thiesmeier *et al.*, 1994).

Differences in reproductive traits may be related to population differences in female body size. Positive correlations between female mass and brood size or larval size were found for *S. s. terrestris* (Thiesmeier, 1992). However, we found no significant within-population correlations between brood characteristics and female traits. In small samples of long-lived, iteroparous animals, such correlations – if they do exist – may be masked by confounding age- or year-specific factors (Kaplan & Salthe, 1979). Female *S. s. gallaica* and *S. s. crespoi* begin reproducing at four years old and may live up to 18 and 12 years, respectively (Rebelo & Caetano, 1997).

The four populations can be arranged from those that invest more in many small larvae to those that invest less in few, large larvae (Fig. 3). When females from populations with different optimal sizes at birth (OSB) allocate the same energy to reproduction (like those from Sintra and Gerês), differences in brood size may emerge, with smaller OSB allowing the production of larger broods, and vice/versa (Roff, 1992).

Metamorphic size was virtually the same in our populations, whether for wild or laboratory-reared larvae. Differences in size at birth are thus probably related to selection acting on larvae in the aquatic environment. Gerês is the only area where strong negative effects of larval drift on survival could be expected, yet this is one of the sites where larval sizes at birth were smaller. Short pond durations could account for the larger larvae from Sintra, but do not explain the smaller larval sizes from Monchique, and especially from Grândola.

Larvae from Sintra were consistently born with a size close to that of viviparous populations from considerably colder climates in northern Spain. There are two historical records of viviparity for *S. s. gallaica*, both from populations located near Coimbra, roughly halfway between Sintra and Gerês (Hillenius, 1996), but this is not a common phenomenon. The Sintra population has some peculiar demographical traits, such as a high proportion of old animals (more than 10 and up to 21 years old) (Rebelo & Caetano, 1997), and a high adult density (from 170 to 500 individuals/ ha) (R. Rebelo, unpubl. data). Smaller broods of larger juveniles have been recorded for old individuals in a number of salamander species (Kaplan & Salthe, 1979). Furthermore, larval fire salamanders are cannibalistic and body size, largely through allometric effects on gape size, determines the possibility of cannibalizing other larvae (Warburg, 1992; Reques & Tejedo, 1996). Sibling cannibalism was observed within all the broods that were kept in captivity. So, perhaps the adoption of large birth sizes is the result of selection for large larvae, able to avoid predation by conspecifics in crowded ponds.

In the laboratory, length of the larval period varied much more than size at metamorphosis, even within each brood, and metamorph sizes were very close to those found in the wild, which suggests that metamorphosis in *S. s. gallaica* is probably more size-determined than age-determined. An initial size difference of half a centimetre corresponded to a two month difference in length of the larval period, but no differences in mean daily growth rate or size at metamorphosis, which is an indication that a small size at birth probably does result in a longer larval period. Given the generally short and unpredictable duration of ponds at Grândola, the striking absence of large birth sizes in this population is puzzling. Here, an interesting parallel may be established between Iberian and Israeli fire salamander populations, which represent the western and eastern limits of the species' distribution. In both cases a large adult body size and the birth of large broods of relatively small larvae were found to occur in dry climates where catastrophic mortality of the larvae due to pond drying occurs frequently. Similarly, in both cases smaller body sizes and smaller broods were found in nearby mesic areas (Degani & Warburg, 1995; this study). For the Israeli populations, Degani & Warburg (1980) found that salamanders from xeric habitats migrate yearly to winter ponds, while those from mesic habitats remain all year near the water sources. This study has found considerably extended parturition periods in Grândola. This may be due to sequential development of several egg cohorts (Dopazo & Alberch, 1994; Sharon *et al.*, 2000). Nevertheless, if females in xeric habitats are mobile and visit different places on different nights, numerous small

larvae may be dispersed among several ponds, which may be a good strategy in areas where such places are often abundant but of unpredictable duration. Similarly, female *Ambystoma annulatum* do not deposit a large proportion of the clutch in one specific location (Hutcherson *et al.*, 1989).

*Salamandra s. infraimmaculata*, the Middle Eastern subspecies of the fire salamander, was recently proposed as a distinct species, apparently having separated from *S. salamandra* between 5 and 13 million years ago (Steinfarz *et al.*, 2000), yet the same responses to mesic and xeric climates persist. Other examples of parallel evolution of reproductive traits are known for the genus *Salamandra*. For example, a viviparous mode of reproduction may have evolved separately at least three times (Steinfarz *et al.*, 2000; Veith, 1994), as an adaptation to high mountain habitats.

Ovoviviparity probably evolved in the fire salamander to counteract larval drift in permanent streams, the main larval habitat in central-European forests (Thiesmeier, 1994; Baumgartner *et al.*, 1999). If that is the case, a return to pond-breeding habits occurred in some marginal habitats, such as mountain plateaus and the Mediterranean ecosystems. The populations we studied, where (with one exception) pond breeding occurs more frequently, still maintain some stream-associated traits – intra-brood coefficients of variation of larval size were very similar, all the broods had normal or leptokurtic size-distributions and there were characteristics consistent with an OSB in some populations. The unpredictable environment of the Mediterranean habitats may be the reason for the greater brood sizes, greater relative brood masses and absence of an OSB in the southern populations.

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## REFERENCES

Baker, J. (1992). Egg production in the smooth newt (*Triturus vulgaris*). *Herpetological Journal* **2**, 90-93.

Barbadillo, L. J. (1987). *La guia de INCAFO de los anfibios y reptiles de la Peninsula Iberica, Islas Baleares y Canarias*. Madrid: INCAFO.

Baumgartner, N., Waringer, A. & Waringer, J. (1999). Hydraulic microdistribution patterns of larval fire salamanders (*Salamandra salamandra salamandra*) in the Weidlingbach near Vienna, Austria. *Freshwater Biology* **41**, 31-41.

Bruce, R. C. (1985). Larval periods, population structure and the effects of stream drift in larvae of the salamanders *Desmognathus quadramaculatus* and *Leurognathus marmoratus* in a southern Appalachian stream. *Copeia* **1985**, 847-854.

C. N. A. (1983). *Atlas do Ambiente*. Lisboa: Direcção-Geral do Ambiente, Ministério do Ambiente e dos Recursos Naturais.

Crump, M. L. (1981). Variation in propagule size as a function of environmental uncertainty for tree frogs. *The American Naturalist* **117**, 727-737.

Degani, G. & Warburg, M. R. (1980). The response to substrate moisture of juvenile and adult *Salamandra salamandra* (L.) (Amphibia, Urodela). *Biology of Behaviour* **5**, 281-290.

Degani, G. & Warburg, M. R. (1995). Variation in brood size and birth rates of *Salamandra salamandra* (Amphibia, Urodela) from different habitats in northern Israel. *Amphibia-Reptilia* **16**, 341-349.

Dopazo, H. & Alberch, P. (1994). Preliminary results on optional viviparity and intrauterine siblicide in *Salamandra salamandra* populations from northern Spain. *Mertensiella* **4**, 125-137.

García-Paris, M., Alcobendas, M. & Alberch, P. (1998). Influence of the Guadalquivir river basin on mitochondrial DNA evolution of *Salamandra salamandra* (Caudata: Salamandridae) from Southern Spain. *Copeia* **1998**, 173-176.

Greven, H. & Guex, G. D. (1994). Structural and physiological aspects of viviparity in *Salamandra salamandra*. *Mertensiella* **4**, 139-160.

Hillenius, D. (1996). Notes on *Salamandra salamandra* ssp. *Bijdragen tot de Dierkunde* **38**, 31-38.

Hutcherson, J. E., Peterson, C. L. & Wilkinson, R. F. (1989). Reproductive and larval biology of *Ambystoma annulatum*. *Journal of Herpetology* **23**, 181-183.

Joly, J., Chesnel, F. & Boujard, D. (1994). Biological adaptations and reproductive strategies in the genus *Salamandra*. *Mertensiella* **4**, 255-269.

Kaplan, R. H. & Cooper, W. S. (1984). The evolution of developmental plasticity in reproductive characteristics: an application of the "adaptive coin-flipping" principle. *The American Naturalist* **123**, 393-410.

Kaplan, R. H. & Salthe, S. N. (1979). The allometry of reproduction: an empirical view in salamanders. *The American Naturalist* **113**, 671-689.

Lizana, M., Ciudad Pizarro, M.J., & Pérez Mellado, V. (1989). Actividad, reproduccion y uso del espacio en una comunidad de anfibios. *Treballs Societat Catalana Ictiologia Herpetologia* **2**, 97-127.

Malkmus, R. (1983). Beschreibung einer neuen Form des Feuersalamanders der Serra de Monchique (Portugal): *Salamandra salamandra (gallaica) crespoi* n. subsp. (Amphibia, Urodela, Salamandridae). *Faunistische Abhandlungen* **10**, 169-174.

McGinley, M. A. (1989). The influence of a positive correlation between clutch size and offspring fitness on the optimal offspring size. *Evolutionary Ecology* **3**, 150-156.

Petranka, J. W. (1984). Breeding migrations, breeding season, clutch size, and oviposition of stream-breeding

*Ambystoma texanum*. *Journal of Herpetology* **18**, 106-112.

Pleguezuelos, J. M. (1997). *Distribución y Biogeografía de los Anfibios y Reptiles en España y Portugal*. Monografías Herpetologicas **3**. Granada: Monografica Tierras del Sur, Universidad de Granada, Asociación Herpetologica Española.

Rebelo, R. & Caetano, M. H. (1997). Body size differences among populations of *Salamandra salamandra* from Portugal: a skeletochronological study. pp. 307-312, in: Bohme, W., Bischoff, W. & Ziegler, T. (Eds): *Herpetologia Bonnensis*. Bonn: (SEH).

Reques, R. & Tejedo, M. (1996). Intraspecific aggressive behaviour in fire salamander larvae (*Salamandra salamandra*): the effects of density and body size. *Herpetological Journal* **6**, 15-19.

Roff, D. A. (1992). *The Evolution of Life Histories - Theory and Analysis*. New York: Chapman & Hall.

Roosenburg, W. M. & Dunham, A. E. (1997). Allocation of reproductive output: egg- and clutch-size variation in the diamondback terrapin. *Copeia* **1997**, 290-297.

Rowe, L. & Ludwig, D. (1991). Size and timing of metamorphosis in complex life cycles: time constraints and variation. *Ecology* **72**, 413-427.

Semlitsch, R. D. (1987). Relationship of pond drying to the reproductive success of the salamander *Ambystoma talpoideum*. *Copeia* **1987**, 61-69.

Sharon, R., Degani, G. & Warburg, M. R. (2000). Ovarian cycle patterns of female *Salamandra salamandra infraimmaculata* in two habitats in northern Israel. *Journal of Herpetology* **34**, 463-465.

Shine, R. (1989). Alternative models for the evolution of offspring size. *The American Naturalist* **134**, 311-317.

Smith, C. C. & Fretwell, S. D. (1974). The optimal balance between size and number of offspring. *The American Naturalist* **108**, 499-506.

Stearns, S. C. (1992). *The evolution of life-histories*. Oxford: Oxford University Press.

Steinfartz, S., Veith, M. & Tautz, D. (2000). Mitochondrial sequence analysis of *Salamandra* taxa suggests old splits of major lineages and postglacial recolonizations of Central Europe from distinct source populations of *Salamandra salamandra*. *Molecular Ecology* **9**, 397-410.

Thiesmeier, B. (1992). *Ökologie des feuersalamanders*. Essen: Westarp Wissenschaften.

Thiesmeier, B. (1994). Aspects of larval ecology of fire salamanders (*Salamandra salamandra*) in Middle Europe. *Mertensiella* **4**, 335-345.

Thiesmeier, B., Sauer, W. & Mutz, T. (1994). Birth and dates of offspring of *Salamandra salamandra bernardezi* form Oviedo. *Mertensiella* **4**, 347-354.

Thorn, R. (1968). *Les salamandres d'Europe d'Asie et d'Afrique du Nord*. Paris: Éditions Paul Lechevalier.

Veith, M. (1994). Morphological, molecular and life history variation in *Salamandra salamandra* (L.). *Mertensiella* **4**, 355-397.

Warburg, M. R. (1992). Breeding patterns in a fringe population of fire salamanders, *Salamandra salamandra*. *Herpetological Journal* **2**, 54-58.

Woodward, B. D. (1982). Local intraspecific variation in clutch parameters in the spotted salamander (*Ambystoma maculatum*). *Copeia* **1982**, 157-160.

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# DO PREDATOR CHEMICAL CUES AFFECT OVIPOSITION SITE SELECTION IN NEWTS?

GERMÁN ORIZAOLA AND FLORENTINO BRAÑA

*Departamento de Biología de Organismos y Sistemas, Universidad de Oviedo, c/ Catedrático Rodrigo Uría s/n, 33071 Oviedo, Spain*

Predation on larval stages has been reported to play an important role in structuring amphibian communities, and for this reason the choice of suitable oviposition places is likely to influence newt fitness. In this study, we assessed whether females of four newt species – marbled newt (*Triturus marmoratus*), alpine newt (*T. alpestris*), palmate newt (*T. helveticus*) and Bosca's newt (*T. boscai*) – avoid chemical cues of predatory brown trout (*Salmo trutta*) in selecting their oviposition site. In laboratory tests, individual females were allowed to choose their oviposition site between places with water conditioned by fish chemicals and others with unconditioned water. *T. marmoratus* females selected preferentially tubs without predator cues as oviposition sites, whereas the other three species did not show significant preference under these conditions. Absence of chemical recognition capabilities, strong philopatry towards oviposition site or predator avoidance based in habitat characteristics are suggested as possible causes of the lack of chemical predator avoidance detected in this experiment.

*Key words*: amphibians, egg-laying, predation, *Triturus*, trout

## INTRODUCTION

Amphibians should choose a breeding habitat that maximizes their fitness by increasing offspring survival, growth and development (Kats & Sih, 1992). Considering the large qualitative differences among breeding ponds and the consequences of this variation for reproductive success, the choice of oviposition habitats is often a much more important factor for fitness than reproductive investment or mate choice (Resetarits, 1996). The value of a potential breeding site in aquatic habitats depends on biotic and abiotic factors, such as pond age (Fegraus & Marsh, 2000), temperature (Seale, 1982), vegetation cover (Wells, 1977), and presence of competitors or predators (Morin, 1983). Some amphibian species have developed toxic or unpalatable substances at egg, larval or adult stages as antipredator protection (Kats *et al.*, 1988; Kiesecker *et al.*, 1996), but in addition to these defences, the capacity to evaluate predator presence can be used to minimize contact with predators, thus increasing survival (Sih, 1987; Kats & Sih, 1992; Hopey & Petranka, 1994). Recognition and behavioural responses to predator cues have been extensively studied in larval amphibians (review in Kats & Dill, 1998; Alford, 1999), but the effects on adult stage responses are less well known (e.g. Joly & Miaud, 1993; Aragón *et al.*, 2000; review in Blaustein, 1999).

Fish predation is possibly the most likely biotic factor influencing the suitability of aquatic sites for use by amphibians and other aquatic organisms with complex life cycles (Kats *et al.*, 1988). Besides, fish introductions are widespread and have been reported as one of the factors associated with severe amphibian declines (Hecnar & McLoskey, 1997; Tyler *et al.*, 1998; Knapp & Matthews, 2000; Gillespie, 2001; Pilliod & Peterson, 2001). For this reason, the aim of this study was to evaluate, in a laboratory experiment, the effect of chemical cues from predatory brown trout (*Salmo trutta*) on the oviposition site selection of adult females of four newt species (*Triturus marmoratus*, *T. alpestris*, *T. helveticus* and *T. boscai*). Chemical cues are more persistent than visual or mechanical cues, so can allow detection of cryptic predators and provide information of past predator presence (Kats & Dill, 1998). In addition, the ability to recognize predators by chemical cues should be advantageous for aquatic prey since it makes predator detection possible in darkness, turbid conditions, physically complex environments or high water volume habitats such as lakes (i.e. in situations in which visual detection is often difficult; Kiesecker *et al.*, 1996; Kats & Dill, 1998). Most previous studies on oviposition site selection were performed with direct presence of free or caged predators (but see Angelon & Petranka, 2002), so that the possible effect of visual or mechanical signals produced by predators could interact with chemical cues.

*Correspondence*: G. Orizaola, Departamento de Biología de Organismos y Sistemas, Universidad de Oviedo, c/ Catedrático Rodrigo Uría s/n, 33071 Oviedo, Spain. *E-mail*: orizaola@correo.uniovi.es

## MATERIALS AND METHODS

### STUDY ANIMALS

Experiments were conducted with adult females of four newt species (marbled newt, *Triturus marmoratus*, $n$=56, snout-vent length, SVL, size range: 74.7-96.3 mm; alpine newt, *T. alpestris*, $n$=33, 53.1-62.0 mm; palmate newt, *T. helveticus*, $n$=66, 37.0-47.9 mm and Bosca's newt, *T. boscai*, $n$=64, 38.0-51.0 mm). *T. marmoratus* is usually found in well-vegetated ponds in lowland areas, *T. alpestris* is a species characteristic of high elevation areas and cool waters, *T. helveticus* appears to be widespread throughout the study area and *T.*

*boscai* is found more often than other newt species in running waters and small first-order reaches where brown trout are sometimes present (Griffiths, 1995; Barbadillo *et al.*, 1999). Female newts were captured by netting in temporary ponds and cattle-watering tanks of central Asturias (northern Spain) during the 1999 and 2000 breeding seasons. Newts were caught in ponds located in all cases more than 500 m away from watercourses, and had probably not experienced predatory fish previously. Females were maintained for a maximum of one week in the laboratory, and then released in their localities of capture, together with the eggs laid during the experimental period. The predator used in the production of chemical cues was the brown trout (*Salmo trutta*), obtained from first generation hatchery fish originating from a local wild stock.

### EXPERIMENTAL PROCEDURES

Pairs of large brown trout (*Salmo trutta*; 25.0 to 35.0 cm fork length, mean±SE = 28.8±1.0 cm) were maintained in a 90 litre tank with dechlorinated and aerated water, and provided the conditioned water (*Predator treatment*). Predators were replaced several times during the experiments and returned to the fish hatchery after use. Trout were not fed to avoid contamination of the water with faeces, and the water was used only after trout had had time to clear their guts. Another 90 litre tank with dechlorinated and aerated water was used to provide unconditioned water (*Predator-free treatment*). Both tanks were located in a room held at constant temperature (10°C). Oviposition responses to fish chemical cues were tested in plastic containers (55 x 40 x 17 cm) each with two plastic tubs (2 litre, 18 cm in diameter) inserted in polystyrene foam. In each container, one randomly selected tub was filled every day with conditioned water (with fish chemicals) and the other was filled with unconditioned water. Water was changed daily to maintain potentially detectable levels of fish chemical cues (Petranka *et al.*, 1987). An artificial oviposition support composed of nine strips of cloth (10 x 0.8 cm) suspended from a float of polystyrene (8 x 5 x 1 cm) was placed in each tub. The experimental design was completed by installing access ramps to the tubs and a refuge outside the water filled with damp moss to prevent the desiccation of the females that did not enter the water. The containers, covered with plastic mesh to avoid the escape of females, were located in a room held at a constant 17°C, and illuminated with fluorescent lights with a LD 12:12 photoperiod. All the containers were checked twice a day (at 0800 and 2000 hrs GMT) for the presence of eggs, which were removed. Females that laid eggs in the first three days were maintained for two days more, and those that did not were excluded from the experiment.

### STATISTICAL ANALYSES

We used binomial tests to test the null hypothesis that the oviposition sites were evenly distributed between treatments (50:50), both for females which laid eggs in only one tub type (with or without fish chemicals; hereafter "selective females"), and for the place of first oviposition for the females which laid eggs in the two tub types ("non-selective females"). Differences between species in the proportion of selective females were tested by a Chi-squared test. We used one-way ANOVAs (with oviposition site as factor) to test for differences in the size of selective females that laid eggs in each tub type. The effect of oviposition site on the number of eggs laid was analysed using a two-way ANCOVA (with species and oviposition site as factors and female SVL as covariate) for selective females, and a repeated-measures ANCOVA for the non-selective females (with SVL as covariate). In all cases, Scheffé tests were used as post-hoc comparison tests. Deviation from normality was tested with Shapiro-Wilk tests and homogeneity of variance with the Bartlett-Box test. We transformed data (square root) when parametric assumptions of normality and homogeneity of variances were not met.

## RESULTS

Most selective *T. marmoratus* females selected the tubs without predator chemical cues to lay their eggs (binomial test, proportion 20:8, $P$=0.018, marginally significant even after applying the Bonferroni correction at $P$=0.072). Selective females of the other species did not select either of the tub types (*T. alpestris* proportion 5:7, $P$=0.387; *T. helveticus* 18:15, $P$=0.364; *T. boscai* 17:20, $P$=0.371; Fig. 1). We did not find significant differences in the selection of the place of first oviposition for the non-selective females of any of the four species (*T. marmoratus* proportion 4:4, $P$=0.636; *T. alpestris* 1:5, $P$=0.109; *T. helveticus* 11:9, $P$=0.412; and *T. boscai* 9:7, $P$=0.402). No differences were found in the proportion of selective females between species ($\chi^2$=2.5, df= 3, $P$=0.481). The size of the selective females was not significantly different between those that laid eggs in

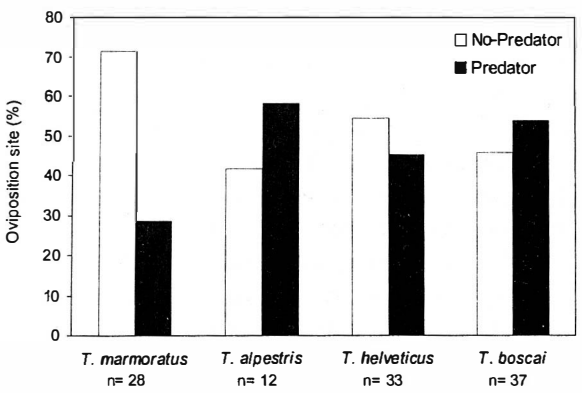


FIG. 1. Oviposition site selection in newts, expressed as the percentage of selective females that used the predator-conditioned or the predator-free tub to oviposit. Total sample size appears under species name.

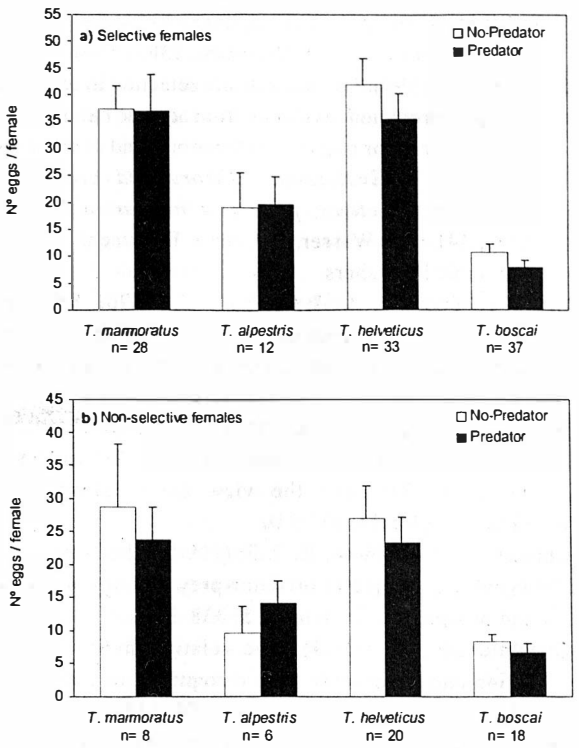


FIG. 2. Number of eggs laid per female newt (mean +SE) in fish-conditioned and unconditioned tubs: (a) females that laid eggs in just one tub type (selective); and (b) females that laid eggs in both tub types (non-selective females). Number of females considered in each case appears under species name.

predator tubs and in non-predator tubs (ANOVAs, $F_{1,24}= 0.158$, $P= 0.694$ for *T. marmoratus*; $F_{1,6}= 2.652$, $P= 0.154$ for *T. alpestris*; $F_{1,28}= 0.640$, $P= 0.430$ for *T. helveticus*; $F_{1,35}= 3.841$, $P= 0.058$ for *T. boscai*). The average number of eggs laid per female in the experimental period was significantly different between species, even considering size-corrected values (ANCOVA: selective: $F_{3,92}=31.5$, $P<0.001$; non-selective: $F_{3,43}=13.1$, $P<0.001$; in both cases *T. marmoratus= T. helveticus> T. alpestris= T. boscai*), but not significantly different between tub types either in the case of selective females (Oviposition place: $F_{1,100}=0.6$, $P=0.449$; Fig. 2a), or non-selective females (Oviposition place: $F_{1,44}= 0.04$, $P=0.831$; Fig. 2b).

## DISCUSSION

Newts frequently use fishless locations as reproductive habitats (e. g. low volume and temporary ponds), avoiding running waters typically occupied by fish (Griffiths, 1995). In the mountain lakes of the study area we have previously reported a strong negative effect of fish presence on amphibian distribution and abundance (Braña *et al.*, 1996). This could be a consequence of direct predation, habitat selection or detection of predators (visual, mechanical or chemical). Chemical senses of olfaction and taste are well developed in *Triturus* species and are important in sexual and feeding behaviours (Cogalniceanu, 1994; Aragón *et al.*, 2000), and in searching aquatic habitats suitable for reproduction (Joly & Miaud, 1993). The results of our experiments indicate that *T. marmoratus* females use fish-predator chemical cues to avoid oviposition in potentially risky situations, selecting non-conditioned tubs in which to lay their eggs. In the case of *T. helveticus*, all the responses were also developed in the direction of predator avoidance but they were not statistically significant. The other two species did not show any preferences between tub types. The results obtained for *T. marmoratus* are consistent with some previous studies that showed modifications of behaviour in prey exposed to predator chemical cues (revision in Kats & Dill, 1998). In particular, predator avoidance in oviposition site selection has been reported for several amphibian species by Resetarits & Wilbur (1989: *Hyla regilla*), Kats & Sih (1992: *Ambystoma barbouri*), Hopey & Petranka (1994: *Rana sylvatica*), Spieler & Linsenmair (1997: *Hoplobatrachus occipitalis*) and Binckley & Resetarits (2002: *Hyla squirella*). In these cases predator avoidance could provide an alternative mechanism to explain the negative spatial association between predatory fish and several amphibian species, which are usually attributed to contemporary predation.

In our experiment, *T. marmoratus* exhibited strong avoidance of predator chemical cues, whereas the other three species did not show any preference. The low number of species used in the experiment prevents us from testing potential ecological and evolutionary correlates of predator avoidance behaviour within a phylogenetic framework. The *cristatus-marmoratus* species group has been signalled as an assemblage of close-related species apart from other newt species, such as *T. alpestris*, *T. helveticus* and *T. boscai* (Busack *et al.* 1988; Zajc & Arntzen, 1999). Because of this separation, *T. marmoratus* exhibit several morphological, physiological and behavioural particularities such as a greater size and a greater use of vegetated habitats (Griffiths, 1995, and personal observations). These characteristics could be associated with a greater use of chemical cues for predator detection, but more studies should be developed to understand the real causes of these differences in antipredator behaviour.

The lack of antipredator avoidance with respect to oviposition site selection and number of eggs laid in other *Triturus* species could also indicate that female newts do not activate antipredator responses using chemical cues associated only with predator presence. This activation could also depend on the simultaneous perception of other cues associated with prey alarm or predator feeding activity (Laurila *et al.*, 1997; Chivers & Smith, 1998). Besides, cues other than chemicals, – such as visual or tactile signals (or some combination of cues) – could be needed to trigger defensive responses. In this regard, Stauffer & Semlitsch (1993) showed that tadpoles of *Rana lessonae* and *R. esculenta* exhibit enhanced responses to fish predator chemical cues, when combined with tactile signals. Otherwise, newt larvae reach metamorphosis only in suitable places (i.e. those with reduced risk of desiccation, competition and preda-

tion), so they could have evolved strong philopatry with respect to oviposition places instead of developing or maintaining detection mechanisms for fish predators that are naturally scarce in their environment (McPeek, 1989; Laurila & Aho, 1997). Also, the avoidance of habitats favourable to fish as oviposition sites could have evolved as the mechanism of predator avoidance, rather than detecting and avoiding the fish itself. In some instances, habitat avoidance could be more effective than predator detection, which is highly dependent on predator temporal and local presence, density or stimulus dilution (Anholt *et al.*, 2000; Van Buskirk & Arioli, 2002). For the species used in this work, this kind of habitat avoidance could be responsible for the use by newts of temporary ponds or water bodies unconnected to streams, as fish populations may not be viable in such habitats. In this scenario fish introductions in naturally fishless habitats (i.e. mountain lakes) can have severe effects on amphibian populations, as has been previously suggested (Terrero 1951; Braña *et al.* 1996). However, further studies are needed to understand the responses of newts to fish presence in more natural situations or when exposed to other types of predator cues.

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## REFERENCES

Alford, R. A. (1999). Ecology: resource use, competition, and predation. In *Tadpoles: the biology of anuran larvae,* 240-278. McDiarmid, R. W. and Altig, R. (eds.). Chicago: The University of Chicago Press.

Angelon K. A. & Petranka, J. W. (2002). Chemicals of predatory mosquitofish (*Gambusia affinis*) influence selection of oviposition site by *Culex* mosquitoes. *Journal of Chemical Ecology* **28**, 797-806.

Anholt, B. R., Werner, E. E. & Skelly, D. K. (2000). Effect of food and predators on the activity of four larval ranid frogs. *Ecology* **81**, 3509-3521.

Aragón, P., López, P. & Martín, J. (2000). Conspecific chemical cues influence pond selection by male newts *Triturus boscai. Copeia* **2000**, 874-878.

Barbadillo, L. J., Lacomba, J. I., Pérez-Mellado, V., Sancho, V. & López-Jurado, L. F. (1999). *Anfibios y reptiles de la Península Ibérica, Baleares y Canarias.* Barcelona: Editorial Planeta.

Binckley, C. A. & Resetarits, W. J. (2002). Reproductive decisions under the threat of predation: squirrel treefrog (*Hyla squirella*) responses to banded sunfish (*Enneacanthus obesus*). *Oecologia* **130**, 157-161.

Blaustein, L. (1999). Oviposition site selection in response to risk of predation: evidence from aquatic habitats and consequences for population dynamics and community structure. In *Evolutionary theory and processes: modern perspectives, papers in honour of Eviatar Nevo,* 441-456. Wasser, S.P. (ed.). Dordrecht: Kluwer Academic Publishers.

Braña, F., Frechilla, L. & Orizaola, G. (1996). Effect of introduced fish on amphibian assemblages in mountain lakes of northern Spain. *Herpetological Journal.* **6**, 145-148.

Busack, S. D., Jericho, B. G., Maxson, L. R. & Uzzell, T. (1988). Evolutionary relationships of salamanders in the genus *Triturus*: the view from immunology. *Herpetologica* **44**, 307-316.

Chivers, D. P. & Smith, R. J. F. (1998). Chemical alarm signalling in aquatic predator-prey systems: a review and prospectus. *Écoscience* **5**, 338-352.

Cogalniceanu, D. (1994). The relative importance of vision and olfaction in mate recognition in male newts (Genus *Triturus*). *Herpetologica* **50**, 344-349.

Fegraus, E. H. & Marsh, D. M. (2000). Are newer ponds better? Pond chemistry, oviposition site selection, and tadpole performance in the Tungara frog, *Physalaemus pustulosus. Journal of Herpetology* **34**, 455-459.

Gillespie, G. R. (2001). The role of introduced trout in the decline of the spotted tree frog (*Litoria spenceri*) in south-eastern Australia. *Biological Conservation* **100**, 187-198.

Griffiths, R. A. (1995). *Newts and salamanders of Europe.* London: T. & A. D. Poyser Ltd.

Hecnar, S. J. & McLoskey, R. T. (1997). The effects of predatory fish on amphibian species richness and distribution. *Biological Conservation* **79**, 123-131.

Hopey, M. E. & Petranka, J. W. (1994). Restriction of wood frog to fish-free habitats: how important is adult choice? *Copeia* **1994**, 1023-1025.

Joly, P. & Miaud, C. (1993). How does a newt find its pond? The role of chemical cues in migrating newts (*Triturus alpestris*). *Ethology, Ecology and Evolution* **5**, 447-455.

Kats, L. B. & Dill, L. M. (1998). The scent of death: chemosensory assessment of predation risk by prey animals. *Écoscience* **5**, 361-394.

Kats, L. B. & Sih. A. (1992). Oviposition site selection and avoidance of fish by streamside salamanders (*Ambystoma barbouri*). *Copeia* **1992**, 468-473

Kats, L. B., Petranka, J. W. & Sih, A. (1988). Antipredator defenses and the persistence of amphibian larvae with fishes. *Ecology* **69**, 1865-1870.

Kiesecker, J. M., Chivers, D. P. & Blaustein, A. R. (1996). The use of chemical cues in predator recognition by western toad tadpoles. *Animal Behaviour* **52**, 1237-1245.

Knapp, R. A. & Matthews, K. R. (2000). Non-native fish introductions and the decline of the mountain yellow-

legged frog within protected areas. *Conservation Biology* **14**, 428-438.

Laurila, A. & Aho, T. (1997). Do female common frogs choose their breeding habitat to avoid predation on tadpoles? *Oikos* **78**, 585-591.

Laurila, A., Kujasalo, J. & Ranta, E. (1997). Different antipredator behaviour in two anuran tadpoles: effects of predator diet. *Behavioural Ecology and Sociobiology* **40**, 329-336.

McPeek, M. A. (1989). Differential dispersal tendencies among *Enallagma* damselflies (Odonata: Coenagrionidae) inhabiting different habitats. *Oikos* **56**, 187-195.

Morin, P. J. (1983). Predation, competition and the composition of larval anuran guilds. *Ecological Monographs* **53**, 119-138.

Petranka, J. W., Kats, L. B. & Sih, A. (1987). Predator-prey interactions among fish and larval amphibians: use of chemical cues to detect predatory fish. *Animal Behaviour* **35**, 420-425.

Pilliod, D. S. & Peterson, C. R. (2001). Local and landscape effects of introduced trout on amphibians in historically fishless watersheds. *Ecosystems* 4, 322-333.

Resetarits, W. J. (1996). Oviposition site choice and life history evolution. *American Zoologist* **36**, 205-215.

Resetarits, W. J. & Wilbur, H. M. (1989). Choice of oviposition site by *Hyla chrysoscelis*: role of predators and competitors. *Ecology* **70**, 220-228.

Seale, D. B. (1982). Physical factors influencing oviposition by the wood frog, *Rana sylvatica*, in Pennsylvania. *Copeia* **1982**, 627-635.

Sih, A. (1987). Predators and prey lifestyles: An evolutionary and ecological overview. In *Predation: Direct and Indirect Impacts on Aquatic Communities*, 203-224. Kerfoot, W. C. and Sih, A. (eds.). Hanover: University Press of New England.

Spieler, M. & Linsenmair, K. E. (1997). Choice of optimal oviposition sites by *Hoplobatrachus occipitalis* (Anura: Ranidae) in an unpredictable and patchy environment. *Oecologia* **109**, 184-199.

Stauffer, H. P. & Semlitsch, R. D. (1993). Effects of visual, chemical and tactile cues of fish on the behavioural responses of tadpoles. *Animal Behaviour* **46**, 355-364.

Terrero, D. (1951). Historia local de piscicultura. *Montes* **39**, 165-171.

Tyler, T., Liss, W. J., Ganio, L. M., Larson, G. L., Hoffman, R., Deimling, E. & Lomnicky, G. (1998). Interactions between introduced trout and larval salamanders (*Ambystoma macrodactylum*) in high-elevation lakes. *Conservation Biology* **12**, 94-105.

Van Buskirk, J. & Arioli, A. (2002). Dosage response of an induced defense: how sensitive are tadpoles to predation risk? *Ecology* **83**, 1580-1585.

Wells, K. D. (1977). Territoriality and male mating success in the green frog (*Rana clamitans*). *Ecology* **58**, 750-762.

Zajc, I. & Arntzen, J. W. (1999). Phylogenetic relationships of the European newts (genus *Triturus*) tested with mitochondrial DNA sequence data. *Contributions to Zoology* **68**, 73-81.

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## SHORT NOTES

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# EUROPEAN POND TORTOISE, *EMYS ORBICULARIS*, NEONATES OVERWINTERING IN THE NEST

SLAWOMIR MITRUS[1] AND MARIA ZEMANEK[2]

[1]*Institute of Environmental Sciences, Jagiellonian University, Gronostajowa 3, 30-387 Kraków, Poland*

[2]*Fiolkowa 7/36, 31-457 Kraków, Poland*

*Key words*: breeding-chamber, hatching, nesting, chelonia

In Central Europe the European pond tortoise, *Emys orbicularis,* is near the northern limit of its range (Fritz, 1998). In this region the tortoise lays eggs from the end of May through to the first half of June (Andreas & Paul, 1998; Jablonski, 1992; Mitrus & Zemanek, 1998; Schneeweiss *et al.*, 1998; Zemanek, 1988). The young tortoises hatch between mid-August and mid-September (Lukina, 1971; Mitrus & Zemanek, 1998, 2000; Schneeweiss *et al.*, 1998). Hatchlings may emerge from the nest by the end of the summer (Schneeweiss *et al.*, 1998; Zemanek & Mitrus, 1997) or even in late October (Mitrus & Zemanek, 2000). Some neonates may overwinter in nests (Kotenko & Fedorchenko, 1993; Servan, 1983 - in Servan, 1998; Schneeweiss & Jablonsky, 2000). However, Bannikov (1951), who studied the tortoise in Dagestan (the north-east of the Caucasus mountain range, south of the Russian Federation) suggested that the neonates overwintering on land did not stay in the nest itself, but buried deeper into the soil.

In 1998 we followed females on their way to the nesting areas and observed them using binoculars. We marked seventeen clutches in the Zwolenka River valley (central Poland). Two of the clutches were destroyed during incubation, nine were dug out on 11 September 1998 as a part of the active protection program (cf. Mitrus & Zemanek, 1998), and the remaining six nests were left for the winter (Table 1).

On 28 March 1999, neonates from the clutch deposited on 29 May 1998 were observed emerging from the soil (Table 1, nest number 7); one neonate was on the surface of the ground about 30 cm from the nest, and a second was in a tunnel from the nest to the surface of ground (Fig. 1). We opened the nest from one side and at the lowest part of the nest (Fig. 2, "C") we found one live hatchling, pieces of eggshell, empty eggshell, and two eggs (probably unfertilized). Above this there was

*Correspondence*: S. Mitrus, Department of Environmental Protection, Faculty of Geology, Geophysics and Environmental Protection, AGH University of Science and Technology, Aleja Mickiewicza 30, 30-059 Kraków, Poland. *E-mail:* emyspl@yahoo.com

FIG. 1. Hatchling of the European pond tortoise (*Emys orbicularis*) during emergence on 28 March 1999, after overwintering in the nest, Zwolenka River valley, central Poland (diameter of the coin = 23.1 mm); photo S. Mitrus.

a layer about 2 cm thick of dense soil (Fig. 2, "B"). Higher still, we observed a second section of the nest (Fig. 2, "A") with one live and eight dead hatchlings. The roof of this chamber was about 5 cm below ground level.

Nine more live neonates were collected later in the Zwolenka River valley. On 3 April 1999, six live and one dead neonates were found in a shallow pit by volun-

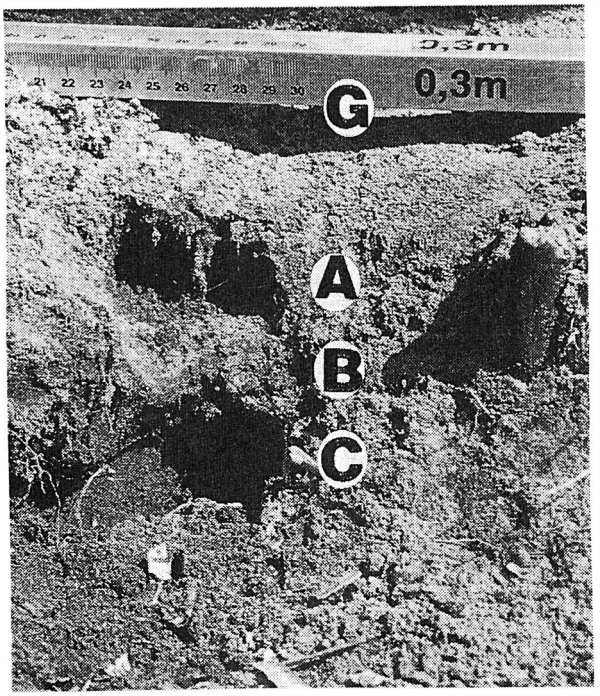


FIG. 2. Section of a nest of *Emys orbicularis* after neonates had overwintered in it. During emergence neonates scrape soil from the roof of the nest and push it down. The chamber is divided into two parts. (G, ground level; A, higher part of the chamber where there were eight dead and one live neonates; B, layer of dense soil; C, the second part of the chamber where were there was one live neonate with pieces of eggshell); photo S. Mitrus.

TABLE 1.History of nests followed over the winter 1998-1999 and ones opened on 11th September 1998.[a] – eggs were counted during oviposition or/and during opening the nest chambers, [b] – number of hatchlings counted from eggshells,[c] – embryos not found, [d] – nests destroyed on summer 1998: one by any predator, and one by agriculture vehicle, [e] – two of them found on the surface of ground (for details see text), [f] – hatched in artificial condition (since 11th to 23rd September 1998), [g] – eggs opened on 18th October 1998, [h] – dead embryos in the last development stages (stages 22-24 – Yntema, 1968);

| Oviposition | | | | Live hatchlings | | | Dead in nest | | Eggs | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Date | No. of laid eggs[a] | Fate of nest | Date of first emergence | Found in nest | Emerged between controls[b] | Hatched from collected eggs | Hatched | Still in eggshells | Dead embryos | Unfert-ilized[c] | Destroyed in nest |
| 20.05.98 | 15 | opened 11.09.98 | | 15 | | | | | | | |
| 26.05.98 | 12/13 | left for overwintering | before 28.03.99 | 0 | 3 or 4 | | 3 | | 1 | 3 | 2 |
| 27.05.98 | 15 | left for overwintering | 28.03-03.04.99 | 6 | 8 | | 1 | | | | |
| 27.05.98 | 12 | opened 11.09.98 | | 10 | | 0 | 0 | | 1[g] | 1[g] | |
| 27.05.98 | 14 | opened 11.09.98 | | 10 | | 0 | 0 | | 0 | 14[g] | |
| 28.05.98 | 11 | opened 11.09.98 | | 3 | | 0 | 0 | | 4[g] | 4 | |
| 29.05.98 | 14 | left for overwintering | 28.03.99 | 4[e] | 0 | | 8 | | 0 | 2 | |
| 29.05.98 | 15 | left for overwintering | 28.03-05.04.99 | 1 | 10 | | 1 | 1 | 0 | 2 | |
| 29.05.98 | 23 | left for overwintering | | | nest not found in spring 1999 | | | | | | |
| 29.05.98 | ? | destroyed[d] | | | | | | | | | ? |
| 29.05.98 | 17 | opened 11.09.98 | | 0 | | 0 | 0 | | 12[g,h] | 5[g] | |
| 29.05.98 | 17 | opened 11.09.98 | | 0 | | 3[f] | 0 | | 7[g] | 7[g] | |
| 30.05.98 | 18 | opened 11.09.98 | | 8 | | 10[f] | 0 | | | | |
| 01.06.98 | 18 | opened 11.09.98 | | 0 | | 1[f] | 4 | | 13[g,h] | 0 | |
| 01.06.98 | 15 | left for overwintering | [opened 16.04.99] | 1 | | | 1 | 10 | 0 | 3 | |
| 02.06.98 | ? | destroyed[d] | | | | | | | | | ? |
| 04.06.98 | 16 | opened 11.09.98 | | 11 | | 0 | 0 | | (2[h]+2)[g] | 1[g] | |

FIG 3. The roof of the nest of *Emys orbicularis* may collapse during the hatchlings' attempts to emerge, revealing neonates in a shallow pit; photo M. Rebis.

teer collaborators (Fig. 3); this pit was formed when the roof of the nest collapsed. Likewise, on 5 April we found one neonate from another clutch. Both nests showed signs that other hatchlings had emerged previously. On 16 April, one live neonate was dug out from a nest chamber and another one was found on a road.

The nest of the European pond tortoise has been described as "pear-shaped" (Zemanek, 1988; Andreas & Paul, 1998). We observed such a shape during the digging behaviour by females and when digging out of chambers in September, as well as during the spring in chambers without hatched eggs. The structure of the breeding chamber after overwintering was different, as described above. We suggest that when neonates try to emerge, they scrape off the soil from the roof of the nest and push it below them - a behaviour described also by Bannikov (1951). This behaviour results in a layer of eggshell fragments and unfertilized eggs that is covered by a layer of soil, and may cause the roof of the nest to collapse (Fig. 3).

Andreas *et al.* (1996) and Schneeweiss *et al.* (1998) wrote about early-season emergence from clutches (in March and April) after overwintering on land. In the Zwoleñka River valley, we found newly emerged hatchlings moving to water on 3 June 1992 and 23 April 1995, and two dead hatchlings on 25 April and 1 May 1995 (Mitrus & Zemanek, 1998; Zemanek, 1992).

Hatchlings of numerous turtle species occurring at lower latitudes in North America overwinter inside the nest chambers (Ultsch, 1989). Such behaviour is probably adaptive because it minimizes the exposure of hatchlings to predators in late summer and autumn, when little growth can be achieved (Gibbons & Nelson, 1975). However, hatchlings of Blanding's turtle *Emydoidea blandingii*, the species most closely related to the European pond tortoise (Burke *et al.*, 1996), seldom overwinter in the nest chambers because freezing is not tolerated by most Blanding's turtle neonates (Packard *et al.*, 2000). The data presented in Table 1 show that most of the hatched European pond turtles that remained in nest chambers for the winter 1998-1999 survived. However, in a nest laid on 1 June 1998 (Table 1, nest No 15), only two turtles hatched before the winter and all those remaining in their eggshells were found dead the following spring.

It is possible that in areas with a continental climate (for example, Dagestan) neonates do not stay in the nest but bury deeper into the soil (Bannikov, 1951), where temperatures during winter are higher, whereas in less severe climates they are able to overwinter in the nest (Kotenko & Fedorchenko, 1993; Servan, 1983 - in Servan, 1998). Our observations demonstrate that at the northern limit of the species' distribution, neonates are able to successfully overwinter in nests, at least in some years.

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## REFERENCES

Andreas, B. & Paul, R. (1998). Clutch size and structure of breeding chambers of *Emys o. orbicularis* in Brandenburg. In Proceedings of EMYS Symposium Dresden 96, Fritz, U. *et al.* (Eds). *Mertensiella* **10**, 29–32.

Andreas, B., Paul, R., Zimmermann, P. & Schneeweiss, N. (1996). Freilandbeobachtungen zum Wanderverhalten frischgeschlüpfter Europäischer Sumpfschildkröten (*Emys orbicularis*) in Brandenburg. *Artenschutzreport* **6**, 34–36.

Bannikov, A. G. (1951). Materialy k poznaniyu biologii kavkazkikh cherepakh. *Uchebnye Zapiski Moskovskogo Gorodskogo Pedagogicheskogo Instituta imeni V. P. Potemkina* **18**, 129–167 (in Russian).

Burke, R. L., Leuteritz, T. E. & Wolf, A.J. (1996). Phylogenetic relationship of emydine turtles. *Herpetologica* **52**, 572–584.

Fritz, U. (1998). Introduction to zoogeography and subspecific differentiation in *Emys orbicularis* (Linnaeus, 1758). In Proceedings of EMYS Symposium Dresden 96, Fritz, U. *et al.* (Eds). *Mertensiella* **10**, 1-27.

Gibbons, J. W. & Nelson, D. H. (1975). The evolutionary significance of delayed emergence from the nest by hatchling turtles. *Evolution* **32**, 297–303.

Jablonski, A. (1992). Próba aktywnej ochrony zolwia blotnego *Emys orbicularis* (L.). In *Czynna ochrona zwierzat*, 107–115. Olaczek, R. & Tomialojc, L. (Eds). Warszawa, PWN.

Kotenko, T. I. & Fedorchenko, A. A. (1993). Reproductive cycle of *Emys orbicularis* in the Danube Delta. In 7th Ordinary General Meeting of the Societas Europaea Herpetologica, Barcelona, Spain. *Abstracts,* 86–86.

Lukina, G. P. (1971). Biologiya razmnosheniya bolotnoj cherepakhy v vostochnom priazov'e. *Ékologiya* **3**, 99–100 (in Russian).

Mitrus, S. & Zemanek, M. (1998). Reproduction of *Emys orbicularis* (L.) in Central Poland. In Proceedings of EMYS Symposium Dresden 96, Fritz, U. *et al.* (Eds), *Mertensiella* **10**, 187–191.

Mitrus, S. & Zemanek, M. (2000). Distribution and biology of *Emys orbicularis* (L.) in Poland. In *Die Europäische Sumpfschildkröte*, Stapfia 69, 149, 107–118. Hödl, W. & Rössler M. (Eds). Linz, Land Oberösterreich, OÖ. Landesmuseums.

Packard, G. C., Packard, M. J. & Lang J. W. (2000). Why hatchlings Blanding's turtles don't overwinter inside their nest. *Herpetologica* **56**, 367–374.

Schneeweiss, N., Andreas, B. & Jendretzke, N. (1998). Reproductive ecology data of the European pond turtle (*Emys o. orbicularis*) in Brandenburg, Northeast Germany. In Proceedings of EMYS Symposium Dresden 96, Fritz, U. *et al.* (Ėds), *Mertensiella* **10**, 227–234.

Schneeweiss, N. & Jablonsky, A. (2000). The reproduction of *Emys orbicularis* in relation to climatic factors in northeast Germany and eastern Poland. In Proceedings of the 2nd Symposium on *Emys orbicularis* – June 1999, Buskirk, J. & Servan, J. (Eds), *Chelonii* **2**, 83–87.

Servan, J. (1998). Ecological study of *Emys orbicularis* in Brenne (Central France). In Proceedings of EMYS Symposium Dresden 96, Fritz, U. *et al.* (Eds), *Mertensiella* **10**, 245–252.

Ultsch, G. R. (1989). Ecology and physiology of hibernation and overwintering among freshwater fishes, turtles, and snakes. *Biological Research* **64**, 435–516.

Yntema, C.L. (1968). A series of stages the embryonic development of *Chelydra serpentina*. *Journal of Morphology* **125**, 219–252.

Zemanek, M. (1988). Skladanie jaj przez zolwie blotne *Emys orbicularis* (L.) w Polsce Srodkowej w warunkach naturalnych. *Przeglad Zoologiczny* **32**, 405–417.

Zemanek, M. (1992). Rezerwat przyrody Borowiec w dolinie Zwoleñki. *Ochrona Przyroda* **50**, 173–195.

Zemanek, M. & Mitrus, S. (1997). Biologia i ochrona zolwia blotnego *Emys orbicularis* w województwie radomskim. *Chronmy Przyrode Ojczysta* **53**, 67–83.

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# THE MATING CALL OF *PELODYTES IBERICUS* (ANURA, PELODYTIDAE)

J. M. PARGANA[1], R. MÁRQUEZ[2,3], R. REQUES[4], M. J. SÁNCHEZ-HERRAIZ[2], M. TEJEDO[4] AND E. G. CRESPO[3]

[1] *Instituto da Conservação da Natureza, Rua General Conde Jorge de Avilez 22, Portalegre P-7300 Portugal*

[2] *Fonoteca Zoológica, Depto. Biodiversidad y Biologia Evolutiva, Museo Nacional de Ciencias Naturales (CSIC), José Gutiérrez Abascal 2, 28006 Madrid, Spain*

[3] *Centro de Biologia Ambiental, Faculdade de Ciências, Universidade de Lisboa, P-1700 Lisboa, Portugal*

[4] *Estación Biológica de Doñana (CSIC). Avda. de María Luisa s/n. Pabellón del Perú. 41013-Sevilla, Spain*

*Key words*: advertisement call, bioacoustics, communication

Acoustic communication is an important feature of anuran social behaviour. In fact, the species-specific mating call is the major pre-mating reproductive isolating mechanism in anurans (Blair, 1958; Gerhardt, 1974; Asquith *et al.*, 1988). Females are attracted almost exclusively to the call of a conspecific male, thus reducing the chances of hybridization. This acoustic specificity provides a tool that has been used extensively to elucidate taxonomic problems. Cryptic species may be identified through the analysis of their mating calls (Crespo *et al.*, 1989; Márquez & Bosch, 1995, 1996).

The Pelodytidae are an ancient family of frogs, originally classified as one genus with two distinct species: *Pelodytes caucasicus* Boulenger (1896) and *P. punctatus* Daudin (1802). The former species is known to occupy the Caucasus region, while the second is distributed over a large area, ranging from the Iberian Peninsula to France, with small extensions to Belgium, Luxembourg and Italy (Van den Elzen, 1975, 1976). Recently, a new species of the family, *P. ibericus,* was described, being endemic to the Iberian Peninsula (Sánchez-Herraiz *et al.*, 2000).

Few studies have analysed the mating call of the Pelodytidae. To our knowledge, the only data referring to the vocalization of *P. caucasicus* are those of Steiner (1968), who described the vocalization of this species as being composed of 2 or 3 multipulsed notes. The acoustic signals of *P. punctatus* have been studied by Hotz (1971) in Liguria (Italy) and by Van den Elzen (1975, 1976) in the Camargue (France). *Pelodytes punctatus* has an advertisement call consisting of two different multi-pulsed notes, named "A" and "B", emitted in a specific order (Van den Elzen, 1975). In the Camargue and Liguria, notes "A" and "B" are emitted in series of pairs "A-B". Both notes can be divided into two separate parts: in the first part, when the pulses increase in intensity (rising phase), the pulse rate is slow. The pulse rate then undergoes a sudden acceleration in the second part of each note, when the pulses decrease in intensity (falling phase).

Paillette *et al.* (1992) analysed the mating calls of a population of *Pelodytes* from the Algarve (southern Portugal) and found a different syntax, as compared with those from the Camargue and Liguria. In the Portuguese population the succession of notes always begins with an "A", but there is frequently more than one "B" in each sequence. The number of "B"s per sequence may be up to seven, which the latter authors attributed to a different geographical dialect.

Differences among Iberian *Pelodytes* species are evident at morphological, anatomical and genetic levels (Sánchez-Herraiz *et al.*, 2000). Before the description of *P. ibericus* as a separate species, some differences relating to mating calls were also found between *P. punctatus* from France and a population of *Pelodytes* from southern Portugal (Paillette *et al.*, 1992). As shown by Paillette *et al.* (1992), these differences include the number of repetitions of "B" notes in a single call sequence, as well as some temporal and spectral features.

In this paper we describe and quantify temporal and spectral characteristics of the advertisement call of males of *P. ibericus*. This bioacoustical information complements the analyses made by Paillette *et al.* (1992) and the description of the new species, which is based on morphological and genetic characters (Sánchez-Herraiz *et al.*, 2000).

Male mating calls were recorded in December 1995 from populations of *P. ibericus* in Mértola (UTM 29SPB16); Almada de Ouro (UTM 29SPB33), southeastern Portugal; and in Córdoba, Southern Spain (UTM 30SUH33). In Portugal, field recordings were made using a Uher 815 microphone connected to a Uher 4000 recorder, at a tape speed of 19 cm/s. In Spain, field recordings were made using an AKG D900 microphone connected to a Sony WM D6C recorder. Air and water temperatures from where the animals were calling were measured immediately after recording, as temperature is the main environmental factor affecting the calls (e.g, Schneider, 1974; Schneider & Nevo, 1972; Gerhardt, 1978; Gerhardt & Mudry, 1980; Paillette, 1986; Crespo, 1981). In the Spanish population, individuals were captured after being recorded and the mass of each (to the nearest 0.5 g) was determined with a Pesola spring balance, and their snout-vent-length (SVL) was determined to the nearest 1 mm by pressing the frog flat against a ruler.

Recordings were processed with Sound Tools hardware (Digidesign Inc.) in an Apple MacIntosh IIfx. Digitization was completed at a sampling frequency of 44.1 kHz and 16 bit resolution in the Spanish population, 22.05 kHz and 8-bit resolution in the Portuguese recordings, using Sound Designer II software (version

*Correspondence:* J. M. Pargana. Instituto da Conservação da Natureza, Rua General Conde Jorge de Avilez 22, Portalegre P-7300 Portugal. *E-mail*: pnssm.parganaj@icn.pt

2.5) and Sound Tools hardware (Digidesign Inc.). A band-pass filter (600-3500 Hz) was used to improve measurements. Signalyze software (version 3.12, Infosignal Inc.) was used to obtain numerical information from waveforms and audiospectrograms. Frequency information was obtained through fast Fourier transformation (FFT) with a width of 3.3 ms and a frequency resolution of 300 Hz.

We analysed a total of 727 calls (2366 notes) from 54 males: 394 calls (1221 notes) from Mértola (15 males) 117 calls (452 notes) from Almada de Ouro (4 males) and 216 calls (693 notes) from Córdoba (35 males).

In a previous, unpublished study that included our two Portuguese populations (Pargana, 1998), a separate analysis was done for each "B" note within a sequence, in order to establish the homogeneity of this note and eliminate the possibility of there being different note types among successive "B" notes. The study concluded that there were no significant differences among "B" notes within a sequence (ANOVA, $P>0.05$ for all variables with the exception of $D_{rising}$: $P=0.02$ and $D_{falling}$: $P=0.039$).

The following call variables were measured, for each note "A" and "B":

Note duration ($D$);
Number of pulses ($Pul$);
Number of pulses in the rising phase of the note ($NP_{rising}$);
Number of pulses in the falling phase of the note ($NP_{falling}$);
Duration of the rising phase of the note ($D_{rising}$);
Duration of the falling phase of the note ($D_{falling}$);
Interpulse duration at the beginning of the note ($IP_{init}$);
Interpulse duration at maximum amplitude ($IP_{max}$);
Interpulse duration at the end of the note ($IP_{end}$);
Dominant frequency at maximum amplitude ($F_{max}$);
Dominant frequency at the end of the note ($F_{end}$);
Duration of the interval between "A" and "B" notes within a sequence (A-B);
Duration of the interval between subsequent "B" notes within a sequence (B-B);
Duration of the interval between calls (B-A).

Within-individual and between-individual coefficients of variation were calculated for all the variables, in order to find out which are "static" and which are "dynamic" (*sensu* Gerhardt, 1991). Static parameters are presumably those that may better characterize the specific call, because they remain fairly constant among individuals from a single population or species, at least within a calling session.

The general structure of the mating call notes of *P. ibericus* was generally similar to that described for the congeneric species *P. punctatus*, being composed of two, different, multi-pulsed notes, which we refer to "A" and "B" to simplify comparisons with the call of *P. punctatus*. The calls were emitted in a specific temporal order, always beginning with an "A", generally followed by one or more "B" notes. The modal number of "B" notes in a sequence was two, emitted in 42% of the sequences from Mértola, 45% of the calls from Almada de Ouro and in 33% of the calls from Cordoba (Table 1). Sometimes there were calls with no "B" notes at all, although these were rare (2% in Mértola, 3% in Almada de Ouro and Córdoba; Table 1). The maximum number of "B" notes in a call was 10, recorded from a male from Córdoba.

A characteristic waveform and audiospectrogram of the call of *P. ibericus* is shown in Fig. 1. The two notes are multi-pulsed, with a change in pulse rate within them, an acceleration occurring immediately after the peak of maximum amplitude; and thus it can be considered that notes have two different parts: a first in which the sound level increases progressively until maximum amplitude is reached and pulses are emitted at relatively wide intervals (rising phase), and a second (falling phase) in which the sound level decreases and the pulse rate increases slightly (Fig. 1).

The numerical parameters (mean, standard deviation and coefficient of variation) for the variables studied are shown in Table 2. Multivariate analysis of variance (MANOVA) using 11 acoustic variables (Table 2) for the Córdoba population showed that both notes, A and B, differed significantly (Wilks' Lambda = 0.0834, $F_{11,58}=57.938$, $P<0.001$). A subsequent discriminant analysis provided 100% correct classification. Univariate analysis of variance (Table 3) revealed that most of variables differed.

Mean duration of note "A" was not significantly different to that of note "B", but note "A" had more pulses. In the "A" note, the duration of the rising phase was shorter, having more or less half the pulses of the falling phase. Although the number of pulses of the rising phase was also a half of the falling phase in the "B" note, the duration of both phases was very similar. Interpulse duration decreased throughout the note; however, within "B" notes it increased until the maximum amplitude peak, decreasing after that.

Concerning the spectral variables, frequency increased throughout both notes, and it was slightly higher in the "B" note. Within the "A" note it ranged from 1.4 to 2.5 kHz at the beginning and from 1.9 to 2.8 kHz at the end, and within the "B" note it ranged from 1.9 to 2.6

TABLE 1. Relative frequency of the number of "B" notes within a sequence (population average of individual relative frequencies).

| Number of "B" notes per call | Almada de Ouro | Mértola | Córdoba |
|---|---|---|---|
| 0 | 0.03 | 0.02 | 0.03 |
| 1 | 0.15 | 0.23 | 0.24 |
| 2 | 0.45 | 0.42 | 0.33 |
| 3 | 0.24 | 0.25 | 0.20 |
| 4 | 0.12 | 0.07 | 0.11 |
| 5 | 0.01 | 0.01 | 0.03 |
| 6 | 0.01 | 0 | 0.03 |
| ≥7 | 0 | 0 | 0.01 |

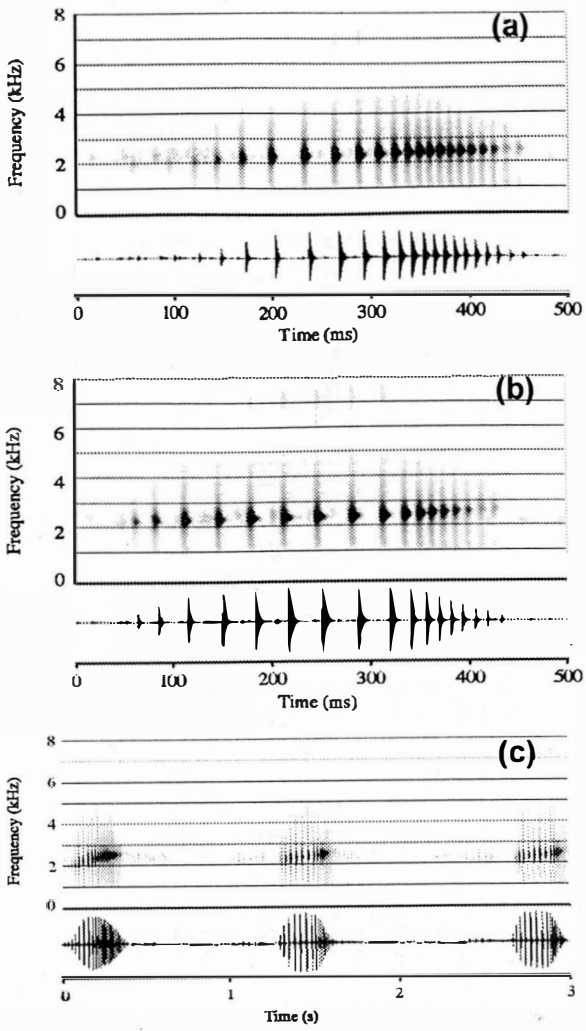


FIG. 1. Audiospectrogram (upper) and oscillogram (lower) of (a) a characteristic "A" note; (b) a characteristic "B" note; (c) a call (A-B-B). The male (SVL 39 mm) was recorded in Córdoba (water temperature 10 °C).

kHz at the beginning and from 2.0 to 2.9 kHz at the end. The increment (upwards frequency sweep between maximum amplitude and the end of the note) was higher in the "A" note than in "B" notes.

The interval between subsequent notes within a sequence was slightly shorter between an "A" and the first "B" than between subsequent "B" notes; it was about 10% of the interval between subsequent calls (Fig 1c).

The relationship between male size and sound parameters was studied in the population from Córdoba, where all recorded males were captured and measured. None of the correlations between male size (SVL) and call parameters was significant.

All the individuals from the samples from Portugal were recorded at similar temperatures (Mértola water temperature range: 14.5-15 °C, Almada de Ouro: all individuals recorded at 16 °C). Therefore, the effect of water temperature on call parameters was measured only in the sample from Córdoba, where the range of water temperatures was 8.4-17.0 °C. With the exception of the interval between calls (B-A), all of the temporal variables (durations, interpulse, and inter-note intervals) and number of pulses were significantly correlated with water temperature. The regressions of mean note duration and number of pulses per note on water temperature are shown in Fig. 2. None of the frequency variables was significantly correlated with water temperature. The number of "B" notes per call was not significantly correlated with temperature.

The within-individual coefficients of variation of dominant frequency were the lowest of all parameters. Call duration and number of pulses per note exhibited a low coefficient of variation. The interval between note "A" and the first note "B" (A-B) also had a low value. The coefficient was higher between subsequent "B" notes and intervals between call sequences (B-A) were highly variable both between and within individuals (Table 2).

Unlike *P. punctatus*, whose mating calls are emitted in series of pairs "A-B" (Hotz, 1971; Van den Elzen, 1975, 1976), *P. ibericus* calls are composed of sequences with a variable number of "B" notes, most often having two "B" notes. None of the individuals recorded emitted only "A-B" calls. Van den Elzen (1976) provides only a rough estimate of the emphasized frequencies for *P. punctatus* for comparison (from 1.8 to 3.5 kHz divided into two bands; one at 1.8-2.1 kHz, and another at 2.8-3.5 kHz), which renders a quantitative comparison with our data impossible. The mean duration of the "A" note for *P. punctatus* from the Camargue (France) was 292.5 ms, and the mean duration of the "B" note was 276.0 ms at 17.5 °C, values that are within the population means from our data (Table 2) but higher than the expected durations for *P. ibericus* at that temperature (Fig. 2a).

Similarly to Paillette *et al.* (1992), who studied a population from Castro Marim (about 10 km away from Almada de Ouro), we found that the most frequent number of "B" notes per call was two, although the former authors found an even higher frequency of sequences with two "B"s (55%). In light of the study by Sánchez-Herraiz *et al.* (2000), and of our own results, we believe that what Paillette *et al.* (1992) considered to be a different dialect of *P. punctatus* from Southern Portugal was in fact the mating call of *P. ibericus*.

Márquez *et al.* (2001) did not find evidence of an increment of the number of "B" notes or call matching through male-male acoustical interactions, suggesting that social interactions are not the main cause of variability in the number of "B" notes.

The variability of the number of "B" notes in a sequence could also be related to a different function of the notes (Narins & Capranica, 1976; Capranica, 1977) or to an increment of informative content through redundancy (Duellman & Trueb, 1986).

Temperature, one of the most important environmental factors affecting call characteristics, may be discarded as a cause. Although temperature is positively correlated with metabolic rate and, consequently, with vocal activity, linear regression did not show any corre-

TABLE 2. Mean and coefficient of variation (CV) of call parameters of *P. Ibericus* for each population studied. "CV between" is a measure of between-individual variability and is calculated with the coefficients of variation of the mean values of the individual males; "CV within" is a measure of the within-individual within-calling period variation, calculated as the average of the individual coefficients of variation of each recording; *n* is the number of individuals. The identification of the acronyms of the call parameters are in the text. Units are ms for duration variables and Hz for frequency variables.

Note "A"

| | | *D* | *NP* | $NP_{raise}$ | $NP_{fall}$ | $D_{raise}$ | $D_{fall}$ | $IP_{init}$ | $IP_{max}$ | $IP_{end}$ | $F_{max}$ | $F_{end}$ | A-B |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Mértola | Mean | 192.6 | 22.7 | 7.5 | 15.1 | 83.3 | 109.4 | 12.7 | 10.9 | 6.1 | 2280.6 | 2424.8 | 489.3 |
| (*n*=15) | $CV_{between}$ | 11.1 | 11.9 | 10.7 | 15.2 | 10.7 | 13.7 | 18.1 | 19.3 | 14.8 | 6.5 | 7.2 | 19.1 |
| | $CV_{within}$ | 4.4 | 5.6 | 10.9 | 8.2 | 11.5 | 8.2 | 11.1 | 15.2 | 7.6 | 2.4 | 2.4 | 13.3 |
| Almada | Mean | 151.4 | 21.4 | 7.5 | 13.9 | 67.5 | 83.9 | 12.2 | 7.9 | 5.3 | 2230.8 | 2364.1 | 426.0 |
| D'0uro | $CV_{between}$ | 7.8 | 21.5 | 18.7 | 24.5 | 3.6 | 12.5 | 30.3 | 30.4 | 34.0 | 4.3 | 6.0 | 5.7 |
| (*n*=4) | $CV_{within}$ | 4.8 | 5.2 | 13.2 | 9.0 | 13.0 | 8.8 | 11.1 | 15.1 | 5.4 | 2.1 | 2.7 | 8.1 |
| Córdoba | mean | 323.6 | 25.5 | 6.4 | 19.0 | 127.7 | 196.4 | 19.4 | 20.1 | 8.9 | 2031.7 | 2348.2 | 755.4 |
| (*n*=35) | $CV_{between}$ | 23.4 | 14.9 | 15.6 | 16.8 | 27.0 | 24.7 | 18.6 | 21.4 | 21.3 | 3.8 | 4.5 | 19.9 |
| | $CV_{within}$ | 7.5 | 10.6 | 11.0 | 12.1 | 15.9 | 12.1 | 14.3 | 19.2 | 9.6 | 3.4 | 1.9 | 15.4 |

Note "B"

| | | *D* | *NP* | $NP_{raise}$ | $NP_{fall}$ | $D_{raise}$ | $D_{fall}$ | $IP_{init}$ | $IP_{max}$ | $IP_{end}$ | $F_{max}$ | $F_{end}$ | B-B | B-A |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Mértola | Mean | 193.5 | 16.3 | 5.5 | 10.8 | 96.4 | 97.2 | 16.3 | 28.3 | 7.8 | 2371.0 | 2481.2 | 546.5 | 7050.7 |
| (*n*=15) | $CV_{between}$ | 8.9 | 14.1 | 10.9 | 17.6 | 10.5 | 14.0 | 17.2 | 19.4 | 19.2 | 7.9 | 8.9 | 18.0 | 46.5 |
| | $CV_{within}$ | 3.9 | 4.2 | 8.8 | 7.3 | 12.4 | 10.6 | 12.6 | 10.7 | 9.2 | 2.0 | 2.7 | 11.7 | 91.0 |
| Almada | Mean | 156.7 | 13.0 | 5.5 | 8.4 | 88.7 | 67.9 | 18.2 | 22.5 | 6.5 | 2349.3 | 2441.9 | 473.9 | 3175.0 |
| D'Ouro | $CV_{between}$ | 7.9 | 20.8 | 16.4 | 26.2 | 10.0 | 24.3 | 36.3 | 11.6 | 27.7 | 6.2 | 6.9 | 8.6 | 39.1 |
| (*n*=4) | $CV_{within}$ | 5.0 | 5.2 | 10.8 | 9.6 | 12.7 | 12.8 | 13.2 | 15.6 | 5.3 | 1.4 | 1.9 | 13.5 | 82.1 |
| Córdoba | Mean | 317.5 | 17.3 | 7.5 | 9.7 | 211.8 | 105.9 | 22.2 | 36.6 | 10.4 | 2128.3 | 2334.0 | 862.7 | 2895.9 |
| (*n*=35) | $CV_{between}$ | 20.9 | 12.1 | 12.0 | 16.5 | 21.5 | 26.4 | 18.0 | 20.8 | 21.2 | 3.8 | 4.9 | 21.0 | 67.2 |
| | $CV_{within}$ | 8.2 | 7.3 | 12.0 | 12.7 | 12.0 | 12.9 | 14.3 | 9.0 | 7.4 | 2.2 | 2.2 | 13.5 | 38.5 |

TABLE 3. Univariate analysis of variance for call variables comparing the notes A and B of *P. ibericus* from the Córdoba population. The estimates that are expressed in bold type remained significant after the sequential Bonferroni correction (Rice, 1989)

| | SS | MS | *F* | *P* |
|---|---|---|---|---|
| *D* | 680.8 | 5062.29 | 0.13 | 0.715 |
| *NP* | 1159.3 | 9.27 | 125.00 | <0.001 |
| $NP_{raise}$ | 21.4 | 0.9 | 23.78 | <0.001 |
| $NP_{fall}$ | 889.8 | 93.85 | 9.48 | <0.01 |
| $D_{raise}$ | 123777.9 | 1645.59 | 75.22 | <0.001 |
| $D_{fall}$ | 143358.5 | 1612.02 | 88.93 | <0.001 |
| $IP_{init}$ | 144.2 | 14.64 | 9.85 | <0.01 |
| $IP_{max}$ | 4740.2 | 38.28 | 123.82 | <0.001 |
| $IP_{end}$ | 34.0 | 4.06 | 8.37 | <0.01 |
| $F_{max}$ | 163606.4 | 6385.56 | 25.62 | <0.001 |
| $F_{end}$ | 3520.8 | 12142.82 | 0.29 | 0.592 |

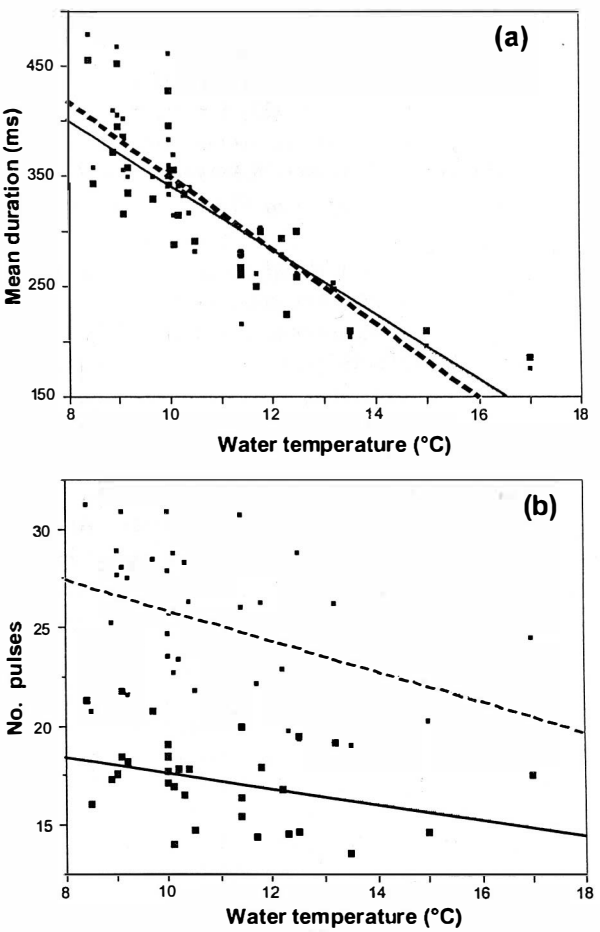


FIG. 2. Effect of water temperature. The dashed regression line and small points are for note A, and the solid line and large points are for note B. (a) linear regression with note duration. Linear fit: note A: Mean Duration (ms) = 686.665 - 33.685 Water Temperature, $R^2$ = 0.700, $P$<0.001, note B: Mean Duration (ms) = 634.895 - 29.412 Water Temperature, $R^2$ = 0.686, $P$<0.001. (b) Linear regression with number of pulses per note. Linear fit: note A, Number of Pulses = 33.623 - 0.773 Water Temperature, $R^2$ = 0.162, $P$=0.0182; note B: Number of Pulses = 21.519 - 0.390 Water Temperature, $R^2$ = 0.101, $P$=0.0376.

lation between this factor and the number of "B" notes per call in Córdoba.

The fact that none of the call variables measured was correlated significantly with male size contrasts with results obtained in other anuran species, where a negative correlation between male size and call frequency variables has been found, with this being determinant for female choice (e.g. Davies & Halliday, 1978; Márquez, 1995) and even for male-male competition (Bee *et al.*, 1999, 2000). However, this may result from the fact that the carrier frequency of the call has such a wide frequency spectrum. The lack of correlation between male size and call frequency variables in *P. ibericus* suggests that if there are any size-related mating trends in this species, these may result from non-static calling parameters such as call intensity, or from mechanisms of male-male competition (e.g. chorus attendance, or fights).

Since patterns of variation in the acoustic properties of mating calls are related to patterns of female choice for the same properties (Gerhardt, 1991), and given the similarity of *P. punctatus* and *P. ibericus* mating calls, we should expect, at least in regions of sympatry, that the females base their choice on fine-tuning properties to identify conspecific males. These properties should have a low coefficient of variation, because it is inversely related to various measures of stereotypy (Gerhardt & Davis, 1988).

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## REFERENCES

Asquith, A., Altig, R. & Zimba, P. (1988). Geographic variation in the mating call of the green tree frog *Hyla cinerea*. *American Midland Naturalist* **119**, 101-110.

Bee, M. A., Perrill, S. A. & Owen, P. C. (1999). Size assessment in simulated territorial encounters between male green frogs (*Rana clamitans*). *Behavioural Ecology and Sociobiology* **45**, 177-184.

Bee, M. A., Perrill, S. A. & Owen, P. C. (2000). Male green frogs lower the pitch of acoustic signals in defense of territories: a possible dishonest signal of size. *Behavioural Ecology* **11**, 169-177.

Blair, F. W. (1958). Mating call in the speciation of anuran amphibians. *American Naturalist* **92**, 27-51.

Capranica, R. R. (1977). Auditory processing of vocal signals in anurans. In *The reproductive biology of amphibians*, 337-355. Taylor, D. H. & Guttman, S. I. (Eds.) New York & London: Plenum Press.

Crespo, E. G. (1981). Contribuição para o conhecimento da biologia dos *Alytes* ibéricos, *Alytes obstetricans*

*boscai* Lataste, 1879 e *Alytes cisternasii* Boscá, 1879 (Amphibia, Salientia) - emissões sonoras. *Arquivos do Museu Bocage* Série C Vol. I nº 3: 57-76.

Crespo, E. G., Oliveira, M. E., Rosa, H. D. & Paillette, M. (1989). Mating calls of the Iberian midwife toads *Alytes obstetricans boscai* and *Alytes cisternasii*. *Bioacoustics* **2**, 1-9.

Davies, N. B. & Halliday, T. R. (1978). Deep croaks and fighting assessment in toads *Bufo bufo*. *Nature* **274**, 683-685.

Duellman, W. E. & Trueb, L. (1986). Vocalization, In *Biology of the Amphibians*, 87-107, New York: McGraw-Hill Book Co.

Gerhardt, H. C. (1974). The significance of some spectral features in mating call recognition in the green treefrog (*Hyla cinerea*). *Journal of Experimental Biology* **61**, 229-241.

Gerhardt, H. C. (1978). Temperature coupling in the vocal communication system of the gray treefrog, *Hyla versicolor*. *Science* **199**, 992-994.

Gerhardt, H. C. (1991). Female mate choice in treefrogs: static and dynamic criteria. *Animal Behaviour* **42**, 615-636.

Gerhardt, H. C. & Davis, M. S. (1988). Variation in the coding of species identity in the advertisement calls of *Litoria verreauxi* (Anura: Hylidae). *Evolution* **42**, 556-565.

Gerhardt, H. C. & Mudry, K. M. (1980). Temperature effects on frequency preferences and mating call frequencies in the green treefrog *Hyla cinerea* (Anura, Hylidae). *Journal of Comparative Physiology* **137**, 1-6.

Hotz, H. (1971). Analyse des paarungsrufes von *Pelodytes punctatus* (Daudin, 1802) (Amphibia – Pelobatidae). *Experientia* **27**, 851-852.

Márquez, R. (1995). Female choice in the midwife toads (*Alytes obstetricans* and *A. cisternasii*). *Behaviour* **132**, 151-161.

Márquez, R. & Bosch, J. (1995). Advertisement calls of the midwife toads *Alytes* (Amphibia, Anura, Discoglossidae) in continental Spain. *Journal of Zoological Systematics and Evoution Research* **33**, 185-192.

Márquez, R. & Bosch, J. (1996). Advertisement call of the midwife toad from the Sierras Béticas *Alytes dickhilleni* Arntzen & García-Paris, 1995 (Amphibia, Anura, Discoglossidae). *Herpetological Journal* **6**, 9-14.

Márquez, R., Pargana, J. M. & Crespo, E. G. (2001). Acoustic competition in male *Pelodytes ibericus*: interactive playback tests. *Copeia* **2001**, 1142-1150.

Narins, P. M. & Capranica, R. R. (1976). Sexual differences in the auditory system of the treefrog *Eleutherodactylus coqui*. *Science* **192**, 378-380.

Paillette, M. (1986). La communication acoustique chez les amphibiens, In *Traité de Zoologie, Amphibiens*, T. XIV (13) Grassé, P. P. (Ed.). 389-416. Paris: Masson.

Paillette. M., Oliveira, M. E., Rosa, H. D. & Crespo, E. G. (1992). Is there a dialect in *Pelodytes punctatus* from southern Portugal? *Amphibia-Reptilia* **13**, 97-108.

Pargana, J. M. 1998. Características espectrais e temporais e correlações genéticas do canto de acasalamento de *Pelodytes punctatus* (Amphibia, Anura). Masters Thesis. Departamento de Biofísica. Universidade de Lisboa. 163 pp.

Rice, W.R. (1989). Analysing tables of statistical tests. *Evolution* **43**, 223-225.

Sánchez-Herraiz, M. J., Barbadillo, L. J., Machordom, A. & Sanchiz, B. (2000). A new species of Pelodytid from the Iberian Peninsula. *Herpetologica* **56**, 105-118.

Schneider, H. (1974). Structure of the mating call and relationships of the European treefrogs (Hylidae, Anura). *Oecologia (Berlin)* **8**, 310-320.

Schneider, H. & Nevo, E. (1972). Bio-acoustic study of the yellow-lemon treefrog, *Hyla arborea savignyi* Audouin. *Zoologische Jahrbücher Abteilung für Allgemeine Zoologie und Physiologie der Tiere* **76**, 497-506.

Steiner, H. M. (1968). *Pelodytes caucasicus* Boulenger, 1896 (Pelobatidae, Amphibia) in der Türkei. *Annalen des Naturhistorischen Museums Wien* **72**, 291-298.

Van den Elzen, P. (1975). Contribution a la conaissance de *Pelodytes punctatus* Daudin (1802) (Amphibia, Pelobatidae), etudié en Camargue. *Bulletin de la Société Zoologique de France* **100**, 691-692.

Van den Elzen, P. (1976). Remarques sur la biologie de *Pelodytes punctatus* Daudin (1802) (Amphibia, Pelobatidae) en Camargue. *Revue française d'Aquariologie* **3**, 99-104.

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Dunson, W. A. (1969*a*). Reptilian salt glands. In *Exocrine glands*, 83–101. Botelho, S. Y., Brooks, F. P. and Shelley, W. B. (Eds). Philadelphia: University of Pennsylvania Press.

Dunson, W. A. (1969*b*). Electrolyte excretion by the salt gland of the Galapagos marine iguana. *American Journal of Physiology* **216**, 995–1002.


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